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Exon Five Deletion

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Estrogen, a key regulator of normal breast growth and differentiation, has been shown to promote both cancer cell proliferation and invasion. This steroid hormone mediates its effect via the estrogen receptor (ER), a member of the nuclear receptor family of transcription factors. A comparison of mRNA ratios of a non-DNA binding estrogen receptor isoform, missing exon 3 (ERΔ3), to the full length ER in breast cancer, cancer cell lines and normal mammary epithelial cells and fibroblasts, revealed a 30 fold reduction of this ratio in cancer cells ( $p<0.001$ ). This suggested a link between the relative loss of ERΔ3 from normal cells and breast carcinogenesis. To directly test its effect on breast cancer cells, stable clones of MCF-7 cells expressing ectopic ERΔ3 protein at levels not exceeding those of physiological ER were generated. In vector transfected controls the ERΔ3-mRNA and protein were less than 10% of total ER while in the ERΔ3-expressing clones, ERΔ3-mRNA and protein represented approximately 50% of the total ER. The presence of ERΔ3 in these cells interfered with both estrogen (E2) stimulation of the pS2 gene mRNA, (inhibited by more than 90% in all ERΔ3-MCF-7 clones as compared with the pMV7 vector transfected control cells), and estrogen mediated down-regulation of its own receptor. Furthermore, analyses of the cells expressing ERΔ3 revealed a reduction in their malignant potential as well as a reversal of several features that distinguish transformed from normal cells. In presence of  $1 \times 10^{-8}$  M E2, compared to control cells, the ERΔ3-expressing cells were density arrested at 50%, and their invasiveness *in vivo* was reduced by up to 79%. As expected, estrogen stimulated anchorage independent growth of both the control pMV7 vector transfected cells and the parental MCF-7 cells, but reduced it to below baseline levels in ERΔ3 clones. In addition, our finding of dome and alveolar-like sphere formation, indicate that cells expressing ERΔ3 may acquire characteristics of normal mammary epithelial cells in culture. Taken together these findings suggest that ERΔ3 may function in normal mammary epithelium to regulate and limit the magnitude of estrogen responses, and that its loss in breast cancer due to an altered splice regulation of ER-mRNA may be a component of breast carcinogenesis.

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1. **I. Erenburg, B. Schachter, R. Mira y Lopez, L. Ossowski** Loss of an estrogen receptor isoform (ER $\delta$ 3) in breast cancer and the consequences of its reexpression: Interference with estrogen-stimulated properties of malignant transformation. 1997 Molecular Endocrinology 11:2004-2015; 1997.

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## **BACKGROUND AND SIGNIFICANCE**

The ovarian steroid hormone estrogen, a key regulator of normal breast growth and differentiation (1), has been shown to promote both cancer cell proliferation and invasion (1, 2). Although conclusive evidence has demonstrated that the estrogen receptor (ER) is the major mediator of estrogen action in both tumor and normal breast (3-7), the precise role of ER in estrogen signaling and its contribution to an altered estrogen response in cancer remains unclear.

### ***Estrogen production during Pubertal development:***

The function of estrogen in breast biology is integrally linked to pubertal development of the reproductive axis, needed for ovarian hormone production (7). The mechanisms underlying the onset of puberty are poorly understood, and are believed to involve the release of neuronal inhibition of gonadotropin-releasing hormone (GnRH), synthesized by the hypothalamus (7, 8). Pulses of circulating gonadotropins (leutinizing hormone (LH) and follicle stimulating hormone (FSH)), produced in response to GnRH, increase in frequency and magnitude with approaching menarche (7). These anterior pituitary hormones in turn stimulate the ovary, leading to estrogen synthesis by the developing follicles (7).

### ***Menstrual cycle:***

As an adult, a woman undergoes a monthly hormonal cycle which lasts an average of 28 days and can be subdivided into two

primary phases, named either to identify ovarian or endometrial physiology: the follicular or proliferative phase (beginning with the first day of menstruation and ending with ovulation), and the luteal or secretory phase (beginning with ovulation and ending with the onset of menses) (7).

Early in the follicular phase, increased FSH production initiates the maturation of a group of slowly growing antral follicles located in the cortex of the ovary (7, 9). In the human, one of these follicles becomes dominant and continues to develop (7), progressing through ovulation. In the growing follicle, the central oocyte is surrounded by cuboidal granulosa cells which in response to FSH both proliferate and differentiate (7, 10). Their maturation is marked by a rise in aromatase activity resulting in the conversion of androgen, which is synthesized by the surrounding interstitial thecal/mesenchymal cells in response to LH, to estrogen (7). As the follicle grows estrogen production increases dramatically, peaking just prior to ovulation (from 60-170ug/day, early follicular phase, to 400-800ug/day, prior to ovulation) (7). This high circulating level of estrogen, sensitizes the gonadotrophs of the anterior pituitary to GnRH stimulation, resulting in a sharp LH surge (7), and ending the follicular phase of the menstrual cycle with ovulation.

In response to the ovulatory surge in gonadotropins, remaining ovarian granulosa and theca cells differentiate by forming a highly vascularized corpus luteum (7), the primary site of both progesterone and estrogen biosynthesis during the luteal phase

(estrogen production remains elevated through most of the luteal phase, 250ug/day, and progesterone production peaks in the mid-luteal phase, 10-40mg/day) (7). If implantation does not occur gonadotropin secretion goes down, resulting in a drop of steroid hormone production, and breakdown of the corpus luteum (7).

***Estrogen effects on pubertal mammary gland development:***

Prior to the onset of puberty in women, the mammary gland consists of approximately 10-20 branching rudimentary ducts which drain into lactiferous ducts, at the nipple (1, 11). These major ducts are lined by a double layer of cuboidal/columnar epithelium, which thins to a single layer at the ends of the branches (11, 12). Surrounding these branches, is a stroma containing a cellular, loose connective tissue matrix (13).

Estrogen rise during puberty initiates mammary growth and differentiation (14, 15). The expansion of mammary tissue, is predominantly attributed to a dramatic increase in fatty stroma (11). Both stromal fibroblasts and ductal epithelial cells proliferate in response to estrogen stimulation, and a branching outgrowth of ductal epithelium into the surrounding fatty tissue ensues (13). Estrogen stimulated epithelial proliferation is especially concentrated at the ends of each branch leading to the development of terminal end bud units (TEBU) (12, 15), the precursors of secretory alveoli in the lobules of a mature lactating mammary gland (14).

Most studies of growth and differentiation of epithelial and stromal cells in response to estrogen have been performed using the rodent model system (13, 15). Estrogen mediates its effect via the estrogen receptor (4), whose expression in mouse mammary fibroblasts has been demonstrated to be approximately 50% of that present in the epithelial compartment (13). In contrast to these findings, studies of ER expression in human mammary tissue, prior to the one presented in this thesis, have been unable to detect ER in human mammary fibroblasts (3), and found only a subset (10-20%) of the normal mammary epithelial cells to be ER positive (3). It is likely that the inconsistency between the rodent and human studies was due to the use of techniques not sensitive enough to detect human ER, present at very low levels in normal human breast. However, it is also conceivable that estrogen affects the stroma indirectly, by stimulating the epithelium to produce growth factors which in turn affect stromal function. Studies demonstrating estrogen stimulation of growth of the mammary epithelium and stroma in both the murine and human models, *in vitro* and *in vivo* (13, 16) do not discriminate between the two possibilities.

As mentioned previously, pubertal expansion of mammary epithelium is accompanied by an estrogen stimulated proliferation of stromal fibroblasts (13, 16). Along the axis of ductal growth, the stromal fibroblasts surrounding the terminal end buds proliferate, defining the interductal space (13). The developing intralobular stroma becomes filled with a matrix rich in hyaluronate (a glycosaminoglycan (GAG) consisting of a chain of unbranched

polysaccharides), sulfated proteoglycans (GAGs covalently linked to a protein core), and tenascin (a matrix glycoprotein) (12, 13, 17). The presence of both hyaluronate and tenascin are believed to be critical in establishing a cell-free space at the edge of outgrowth allowing for the migration of developing end buds into the mammary fat pad (13, 16). As the mammary gland reaches maturity and epithelial growth and morphogenesis cease, a loose intralobular matrix rich in fibroblasts, occasional lymphocytes, blood vessels, and a mesh of porous matrix proteins (ideal for passage and storage of hormones and nutrients) is established (11, 13).

Unlike the loose intralobular stroma described above, the matrix that separates lobes of the mammary gland, the interlobular stroma is dense, rich in collagen fibers, and contains fewer fibroblasts (13). It is in this space that intervening adipose tissue expands during puberty (13), helping to establish the proper architecture of the gland during branching morphogenesis and substantially adding to the observed breast growth (11).

#### *The adult non-lactating mammary gland structure:*

The adult breast consists of a branched mammary tree containing a network of ducts which interconnect lobules of the tubulo-alveolar gland (1, 11, 18). The non-lactating lobules and ducts are lined by a combined epithelium of cuboidal/columnar luminal cells and basal myoepithelial cells (9, 11). Although in histologic analysis the luminal cells appear as a layer above the myoepithelium, in fact, both types of epithelial cells are in direct

contact with the basement membrane, which separates them from the underlying stroma (11).

Both morphology and function distinguish these mammary epithelial cells from one another. The luminal epithelium, the secretory component of the glands, responds to lactogenic hormones of pregnancy by producing milk and releasing it into the lumen of the glands via apocrine secretion (14, 19). Concurrently, initiation of lactation stimulates the myoepithelial cells to constrict the lobules and ducts, via the contraction of muscle-like (smooth muscle actin) filaments located in long cytoplasmic processes surrounding the glands, thus aiding in milk ejection (9, 11, 18).

Besides their functional distinctions, specific cellular markers differentiate between these luminal and basal cells (20). Intermediate filaments, a diverse family of structural proteins, are commonly used in the identification of different epithelial cell populations (17). In the mammary gland, one such class of proteins, the cytokeratins, have been extensively used to determine basal or luminal cell origin (20). In the basal epithelium, the myoepithelial cells express cytokeratins 5 (K5), 14 (K14), and 17 (K17), as well as the intermediate filament protein vimentin (20); whereas, luminal epithelial cells can be identified by cytokeratins 7 (K7), 8 (K8), 18 (K18) and 19 (K19) (20). In addition to the intracellular cytokeratin markers, cell surface molecules such as common acute lymphoblastic leukemia antigen (CALLA), on the basal epithelium (21), and polymorphic epithelial mucin (PEM), on the luminal cells (21), permit

the immunoselection of specific cell populations of the mammary epithelium. Using this complement of cytokeratin and cell surface markers, myoepithelial and luminal cells can be independently evaluated either by assessing such marker expression *in situ* or by determining the content of coding mRNA or protein, using northern or western blotting techniques.

These distinguishing markers have not only enabled researchers to discriminate between mammary epithelial cells, both *in vivo* and in primary cell cultures *in vitro* (20), but has lead to the identification of a minor component of epithelial cells which may represent cells with an increased proliferative potential, possibly the stem cell population of the mammary gland (20, 22). The presence of such progenitor cells is suggested by studies of normal isolated epithelial cells in culture (20, 22). Unlike the majority of the glandular epithelial cells, which senesce within approximately four passages, a morphologically distinct component of the mammary epithelium can continue to grow in culture for up to twenty passages (20, 22). Analysis of these cells with high proliferative potential revealed that they contain cytokeratin markers consistent with both luminal and myoepithelial phenotypes (20), perhaps giving rise to both cell types during differentiation. Future studies of this putative class of progenitor cells will clarify the factors and events governing normal mammary differentiation, and may play a critical role in our understanding of the inappropriate changes that occur during malignant transformation.

In addition to the glandular epithelium, stromal fibroblasts play an integral role in the compartmentalization of the mature breast. These cells maintain the extracellular matrix (ECM) of the mammary gland, which has been demonstrated to be a critical regulator of both epithelial cell growth and differentiation (13, 16). A small subset of stromal cells, called myofibroblasts, are interspersed around the lobules and surrounding the primary ducts (23). Like myoepithelial cells, these cells contain contractile filaments which aid in milk ejection (11). Although under normal conditions these cells are a substantial minority of the total fibroblast population, in several pathologic states, either surrounding a cancerous lesion or at a site of acute injury, they constitute the majority of stromal cells (23). Not surprising, given the well documented function of such cells in wound healing and inflammatory responses in other systems (24), myofibroblasts in breast pathology are believed to arise from the specific recruitment, proliferation and differentiation of the resident fibroblast population (23).

*Estrogen effects on the normal adult mammary gland:*

Estrogen, produced during an adult woman's menstrual cycle, is a critical regulator of the cyclical growth and regression of the mammary gland (11). When estrogen levels increase, during the proliferative phase, an increase in DNA replication is observed in ductal mammary epithelium (14). During the secretory phase of the cycle, the combined actions of estrogen and progesterone initiate epithelial proliferation in the terminal end buds (15, 20).

Specifically, this proliferation is mostly observed in the luminal epithelium with little growth of the basal cells (20, 25). By the end of the secretory phase, terminal end bud/alveolar units begin to differentiate, with secretory material observed in the lumens of some glands (11). However, in the absence of continued production of both estrogen and progesterone, which occurs during pregnancy, this secretory material is quickly resorbed (11), and a drop in hormone levels results in the apoptotic death of a subset of mammary epithelial cells (11), similar to that observed during mammary gland post-lactational involution (9, 14).

Stromal cells are also responsive to the effects of estrogen (13, 16). An increase in the mitotic index is observed during the high estrogen phase, although these effects are not as dramatic as that in the glandular epithelium (13, 16). During the course of the menstrual cycle morphological changes occur in the stroma. Towards the end of the secretory phase, a marked edematous stroma containing an increased number of neutrophils and swollen fibroblasts is often observed (11).

Although the effects of estrogen *in vivo* have been well established, in order to delineate its action on individual cell types of breast tissue, reduction mammoplasty specimens, providing both cultured stromal fibroblasts and epithelial cells, have been used (13, 22). Surprisingly, neither purified mammary epithelial cells nor purified mammary stromal fibroblasts alone were growth stimulated by physiologic levels of estrogen *in vitro* (13). In fact, when growing

normal epithelial cells are confronted with stromal fibroblasts in the absence of estrogen their growth is inhibited (26). Only with the addition of estrogen to a co-culture of epithelial and fibroblast cells, in the presence of extracellular matrix (ECM), is estrogen induced proliferation of both cell types observed (13). Furthermore, such estrogen stimulation of co-cultures results in the induction of progesterone receptor (an estrogen responsive gene) in the epithelial cells, but not in the fibroblasts (27), demonstrating cell type specificity of different estrogenic effects. These studies demonstrate that complex stromal/epithelial interactions are tightly coupled to estrogen regulation of normal mammary cells.

### ***Breast Cancer Biology***

As with most cancers, the development of breast cancer is believed to be a multi-step process (1, 2). Infiltrating ductal carcinoma accounts for the majority of breast cancer cases (~80%), with lobular, medullary, mucinous, and papillary carcinomas representing the remainder of observed phenotypes (1, 2, 11). The stepwise progression to breast cancer begins with benign proliferation of ductal epithelium, termed ductal hyperplasia, which progresses to an atypical hyperplasia (2); both considered non- or pre-cancerous lesions. The latter is often difficult to distinguish pathologically from the next stage in cancer progression, carcinoma *in situ* (11). Ductal carcinoma *in situ* (DCIS) is marked by extensive intraductal proliferation of the epithelium, and is commonly characterized by large pleomorphic cells containing irregularly sized nuclei with frequent mitotic figures, surrounded by an intact basal

lamina (1, 2, 11). As these cancer cells acquire the ability to break down the basement membrane and invade the underlying vascularized stroma, the resultant infiltrating ductal adenocarcinoma progressively acquires a more aggressive phenotype capable of transversing blood vessels (1, 2, 11). Metastatic dissemination of cancer cells throughout the body, via the vasculature and lymphatics, to sites such as bone, liver and brain, and, in later stages, to most visceral organs, ultimately leads to death (1, 2).

Early detection of breast cancer, due to the advent of mammography, has been an important advance in management of this disease (1, 2). The fact that 20-30% of patients who present with lymph node negative disease, die of recurrence within 10 years of diagnosis (1, 2), indicates that dissemination of breast cancer cells can occur when the tumor is still small (size < 1cm). Given that the onset of metastatic disease occurs early in disease progression, our understanding of the events that initiate breast cancer is critical for both its treatment and eventual prevention.

***Estrogen exposure and breast cancer:***

Estrogen is a well established mitogen in breast cancer (1, 2, 28). Epidemiologic studies have identified a number of factors which taken together suggest that the cumulative exposure to estrogen over the course of a woman's life significantly increases her chances of developing breast cancer (1, 2). Some of the primary factors contributing to such an increase in risk include: early age at menarche, late menopause, nulliparity, first trimester abortions -

either spontaneous or therapeutic, obesity, post-menopausal estrogen replacement therapy, and possibly long term use of oral contraceptives (1, 2). All of these factors increase lifetime exposure of the mammary gland to estrogen, which in turn strongly correlates with an increased risk of breast cancer in women. In addition, although the highest incidence of breast cancer is observed in post-menopausal women, this is largely due to a steep rate of increase in age-specific incidence of the disease throughout the reproductive years; which drops off, reaching a plateau in post-menopausal women (2). Taken together, these data suggest that exposure of mammary tissue to estrogen produced during the reproductive years contributes to the etiology of breast cancer.

***ER expression in breast cancer:***

Currently, all breast tumors, in addition to being clinically and pathologically staged, are also assayed for the presence of estrogen (ER) and progesterone (PR) receptors (2, 11). ER/PR positivity, associated with a more differentiated phenotype, is an indicator of good prognosis, and occurs in approximately 60% of all breast tumors (2). The mitogenic action of estrogen on breast cancer has led to the widespread use of the anti-estrogen, tamoxifen, in adjuvant therapy, second only to chemotherapy (29, 30). ER expression in breast tumors is the best predictor of tumor responsiveness to tamoxifen therapy (2, 29, 30); in fact, more than 70% of ER+ breast tumors regress during tamoxifen treatment, whereas only 5-10% of ER- tumors show this favorable response (2, 29, 30).

The elevated level of ER in tumors, much higher when compared to normal mammary tissue, is generally a result of ER gene over-expression (2, 30). The suggestion that a gain of ER expression occurs during the early stages of breast cancer is supported by studies showing that a proportion of ductal carcinoma *in situ*'s are ER positive (31). The elucidation of the factors that govern ER gene expression *in vivo* may provide important insights into the role of ER in disease progression.

Analysis of estrogen signaling in breast cancer has shown that the mitogenic action of estrogen through ER is largely mediated by the induction of local autocrine growth factors (1, 2, 4, 28, 30, 32-35), their receptors (such as TGF $\alpha$ , IGF-1, and EGF-R) (1, 2, 4, 28, 30, 32-35), as well as critical cell cycle genes (such as myc, cyclin D1, and the fos/jun family of transcription factors) (2, 4, 35-43). These same estrogen target genes are often mutated or amplified in breast tumors, resulting in a growth advantage for these tumor cells. Perhaps the acquisition of such growth signals, normally downstream of estrogen, abolishes the cell's need for hormone and, consequently, ER. In fact, loss of ER may be selected for during the late stages of disease progression in order to circumvent some of estrogen's differentiative functions (such as PR induction).

#### *Cell lines as models of breast cancer:*

Breast cancer cell lines have provided an invaluable tool in the study of estrogen signaling in breast cancer. Most breast cancer cell lines, derived from highly malignant tumors, were established either

from metastases or from pleural effusions (20). These immortalized cancer cells share many characteristics with primary breast tumors, both in their phenotype and responsiveness to estrogens and tamoxifen (20, 28).

Analyses of breast cancers have shown that the majority of tumors (>90%), including most of the ER+, express cytokeratin markers consistent with the luminal epithelium, K8, K18 and K19 (20). However, it remains unclear whether this is because the luminal epithelium is more susceptible to oncogenic transformation or because a carcinogenic event targets stem cells during luminal differentiation. A smaller fraction of very aggressive, almost exclusively ER- tumors (9% of Grade II and 35% of Grade III or above) express markers of the basal mammary cells, K14 and vimentin in addition to K8, K18 and K19 (20). Such cancers have a poor prognosis and are less differentiated (2); and arise either from progressive de-differentiation of a less aggressive luminal phenotype, or directly from the transformation of a mammary stem cell. Similarly, several ER+ breast cancer cell lines (such as MCF-7, T47D, ZR-75) express exclusively the cytokeratin (K8/K18/K19) markers of the luminal epithelium (20), whereas ER- breast cancer cell lines (such as MDA-MB-231, Hs 578T) express both cytokeratin (K14) and vimentin filaments in addition to K8, K18, and K19, consistent with the basal cells (20).

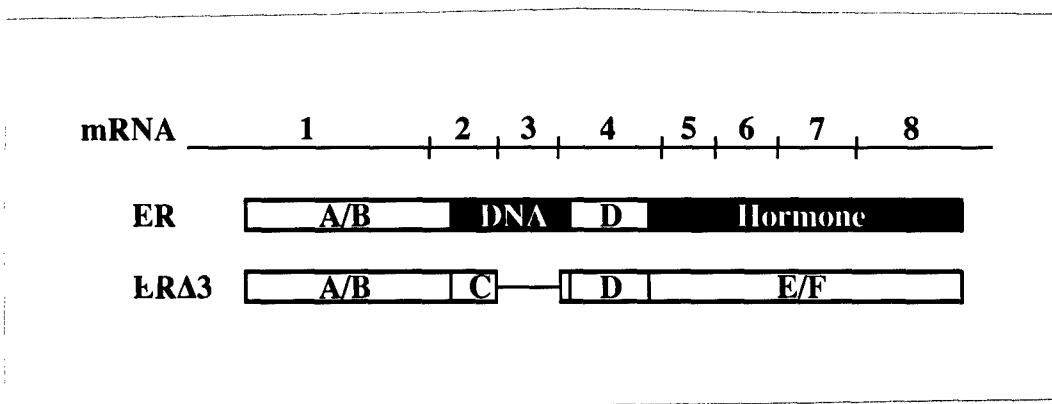
The ER+ breast cancer cell lines have been critical in delineating the effects of estrogen on different parameters of cancer cell growth

and invasion (1, 28). As described previously, estrogen stimulated proliferation of ER+ breast cancer cells is in part mediated by the induction of growth factors and their receptors (such as TGF- $\alpha$ ; EGF-R; IGF-1) (1, 2, 4, 28, 30, 32, 33, 35), and inhibition of growth suppressors (such as TGF- $\beta$ ) (34). In addition, estrogen regulation of proteins critical for proliferation (such as cyclin D1, myc, and the fos/jun family of transcription factors) is an important component in its effect on cancer cell growth (37, 39, 41, 44-46). Estrogen stimulation of breast cancer cell proliferation is well demonstrated both *in vitro*, in anchorage dependent and independent growth (2, 28, 37, 39, 41, 44-46) (an indicator of tumorigenic potential), and *in vivo* in tumor formation in nude mice (47) (a model used to assess both growth and invasive/metastatic potential of cancer cells). In addition to its effect on growth, estrogen stimulation of cancer cell invasion is likely mediated by the induction of several proteolytic enzymes (such as plasminogen activators, cathepsin D, and collagenase IV) (16, 48-52), shown to be involved in the degradation of extracellular matrix (49, 50, 52).

Of the estrogen responsive breast cancer cell lines, perhaps the most studied is the MCF-7 line. These cells contain a high level of ER (30), relative to most primary ER+ breast tumors and breast tumor cell lines, and their growth is stimulated by estrogen and inhibited by tamoxifen, both *in vivo* and *in vitro* (53-55). In addition, its intermediate malignant characteristics make this cell line convenient for observing either stimulatory or inhibitory effects both at the phenotypic and molecular level.

*Structure and function of the estrogen receptor:*

The estrogen receptor belongs to a family of nuclear steroid hormone receptors that regulate gene transcription as ligand bound dimers (4, 30, 33, 56). Like other members of this family, ER contains distinct functional domains: an amino terminal transactivating domain (A/B), a DNA binding domain, containing two zinc fingers (C), a hinge region, containing four nuclear localization signals (D), and a carboxy terminal hormone binding and hormone dependent transactivation domain (E/F) (Fig. 1) (4, 57, 58).

**Figure 1**

**Figure 1*****Diagram of ER and ERΔ3 mRNA and protein structures.***

Structural domains of ER and ERΔ3 protein and corresponding coding exons of the ER mRNA. An amino terminal activation function 1 (AF-1) domain, encoded by exons 1 and 2 (A/B); a zinc finger DNA binding domain, encoded by exon 2 (first zinc finger) and exon 3 (second zinc finger) (C); a hinge region containing several nuclear localization signals, encoded by exon 4 (D); and a carboxy terminal hormone binding domain also containing the activation function-2 (AF-2), encoded by exons 5-8 (E/F).

In the absence of hormone, predominantly nuclear ER exists in an oligomeric complex with several heat shock proteins, (hsp90 and hsp70), co-chaperones (p60 and Hip or p48), and accessory proteins (an immunophilin and p23), which keep the receptor inactive in the absence of hormone (56, 59-61). Recent evidence suggests that the proper assembly of this complex, mediated by some of its components, is necessary for the conformational maturation of the receptor (61). This hsp containing complex can regulate the availability of ER in a cell by binding to the unliganded receptor and stabilizing the ligand binding pocket in a conformation permissive for hormone binding (61). Upon ligand binding, this complex dissociates and receptor dimers bind to DNA, resulting in estrogen signaling (56).

***ER regulation of gene expression:***

Transcriptional regulation by ER is mediated by two regions of the receptor: activation function-1 (AF-1), located in the amino terminus (A/B), and activation function-2 (AF-2) located in the carboxy terminus (E/F) (56, 62, 63). The estrogen dependent AF-2, by interacting with AF-1, induces a class of genes that are sensitive to inhibition by tamoxifen (56, 62, 63). When bound to an agonist the ER AF2 domain can interact with other transcriptional regulators leading to productive gene expression (64). In contrast, an antagonist bound receptor, does not confer the appropriate AF2 conformation needed to stimulate such genes (58, 65). Such receptor recognition of agonist versus antagonist is believed to be mediated via the F domain, located in the carboxy terminal portion of the protein (66).

Although, the two independent AF's cooperate and enhance each other's transactivation activity (65), their individual contributions are promoter and cell type specific (58, 64, 65). AF-1 regulation of gene expression is ligand-type independent, and receptor activation can be mediated either by estrogen, tamoxifen, or ER phosphorylation (a non-ligand binding mechanism) (58, 65, 67-69); furthermore its effect is strongest in cells in which AF-2 action is weak (58, 65).

ER regulation of gene expression is mediated by at least two different pathways: ER binding to palindromic DNA sequences, termed ERE's, in the promoter regions of estrogen responsive genes (70, 71); and, alternatively, through DNA binding domain independent, protein/protein interaction of ER with the fos/jun class of transcription factors (46).

***ER action mediated by estrogen response elements:***

The classic estrogen regulation of ERE containing genes requires receptor dimerization (57, 72). The primary dimerization region of ER is mapped to the hormone binding domain (57), with the second zinc finger of the DNA binding domain also implicated in a weak dimerization function (57). When estrogen is present, ER binds ERE's with high affinity, and, by interacting with the basal transcription machinery of the cell, induces or represses gene expression (58, 72, 73). The stability of ER interaction with DNA is influenced not only by the native promoter context (74, 75), but also by its interactions

with accessory proteins, which act to either activate (co-activators) or repress (co-repressors) hormone dependent gene expression (76-79). The presence and availability of these accessory factors may play an integral role in determining the final outcome of estrogen action on cells of different origin.

Since the description of the vitellogenin gene consensus ERE sequence, similar elements have been identified in many estrogen responsive genes (32, 70, 71). However, as is often the case, the vast majority of such ER regulated genes do not contain "perfect" ERE's. Instead, single or multiple imperfect palindromes with various mismatches (such as the pS2 gene - two imperfect ERE's) (54, 80), or half palindromic sequences separated by long stretches of DNA (such as the PR gene) (4), mediate ER responses. The effect of estrogen on such promoters can be blocked by the addition of anti-estrogens, tamoxifen or ICI 164,384.

These two antagonists have a distinctly different mechanism of action. Tamoxifen bound ER (tamoxifen competes with estrogen for the ligand binding domain) allows the receptor to bind DNA with high affinity, but keeps AF-2 in an inactive conformation, thereby inhibiting ERE dependent gene expression (29, 65). Alternatively, ICI 164,384 disrupts ER dimerization by steric hindrance (the dimerization domain and the binding pocket are both present in the E/F region of the protein) and increases the rate of turnover of the receptor (81). Unlike tamoxifen, by inhibiting ER dimerization, ICI

164,384 abolishes high affinity DNA binding, perhaps explaining its action as a 'pure' antagonist (58, 65).

***ER action mediated by protein/protein interaction:***

The second mechanism by which ER modulates gene expression is by protein-protein interaction with the components of the AP-1 complex, namely the fos/jun transcription factors (46). Direct interaction of ER with jun, via the amino-terminal (A/B) domain (46), may account for some of the ligand independent functions of AF-1. Estrogen bound ER synergizes with the AP-1 complex to stimulate AP-1 dependent gene transcription in cells of breast, endometrial, and cervical origin (46). As expected, tamoxifen, an antiestrogen in the breast, does not show such agonistic activity in mammary epithelial cells, whereas it is capable of efficiently stimulating an AP-1 reporter in endometrial and cervical cells (46). Factors that control this differential effect of tamoxifen remain to be elucidated, however these results suggest that some of the "estrogenic" actions of tamoxifen, observed in the endometrium and cervix, may be attributed to its stimulation of AP-1 regulated genes.

Importantly, deletion of the ER DNA binding domain does not alter estrogen induction of the fos/jun regulated AP-1 promoter, supporting the notion that estrogen regulation of such genes is independent of ER binding to DNA, and independent of the ER DNA binding domain (46). As expected, the promoters of estrogen responsive genes are complex, containing multiple transcription factor sites in the same regulatory region. The cooperativity or

competition of transcription factors (such as ER or fos/jun), their availability in a particular cell type, and the stability of their interactions with the DNA, accessory factors, or each other, all impact on the magnitude and specificity of estrogen regulated gene expression *in vivo*.

***Estrogen receptor diversity:***

Since the original cloning and characterization of the estrogen receptor (82) (when only one form of the receptor was believed to exist), the understanding of estrogen action through ER, has been increasingly complicated by the discovery of receptor isoforms generated by alternative splicing of ER mRNA (soon to be termed ER $\alpha$ ), and the more recent identification of a new gene (ER $\beta$ ) encoding another form of the receptor. Such diversity of ER isoforms parallels other members of this receptor super-family (63, 73, 83, 84), and their varied functions in estrogen responsive tissues await further exploration.

[For the sake of consistency with the existing literature, ER $\alpha$  will be referred to as ER.]

ER $\beta$ , recently cloned from human (85), rat (86, 87) and mouse tissues (88), is classified as a estrogen receptor by both homology to ER (through most of the DNA, approximately 95%, and hormone binding, approximately 60%, domains with almost complete identity of the amino acids in the ligand binding pocket), and its affinity to estrogen (comparable to that of ER) (85, 87, 88). In response to

estrogen, ER $\beta$  can bind and transactivate both an ERE linked to a reporter gene, and an endogenous estrogen responsive gene, at levels comparable to that observed for ER. Although it shares similar regulatory pathways with ER, such as activation by phosphorylation and interaction with a common coactivator (88), given its unique amino terminus, ER $\beta$  is likely to possess distinct AF-1 regulated gene targets. In addition, its tissue distribution suggests that it is not likely to be a redundant receptor in estrogen signaling, since expression of ER $\beta$  is highest in the ovaries, prostate and testis, as well as in the spleen, and thymus (85, 86, 88-90). In contrast to ER, this receptor has not been detected in breast cancer cell lines (89, 90), although its expression in primary breast tissue remains to be determined. Evidence of gonadotropin regulation of ER $\beta$  levels in ovarian follicles (86), further implicates it as a critical form of the estrogen receptor, important in reproductive physiology.

Although it is the newest member of the estrogen receptor family, ER $\beta$  is not the only one that has received much recent attention. Alternatively spliced ER-mRNA's were first noted in breast tumors and tumor cell lines, and suggestions of aberrant ER isoforms in breast cancer etiology were made without appropriate comparison with normal breast tissue (91-114). To date mRNA isoforms missing exon 2 (ER $\Delta$ 2), exon 3 (ER $\Delta$ 3), exon 4 (ER $\Delta$ 4), exon 5 (ER $\Delta$ 5), and exon 7 (ER $\Delta$ 7), as well as several mRNAs with multiple deleted exons have been identified (91-114). Initial studies have shown that skipping of exons 2, 5, and 7 create a frame shift that leads to premature termination of translation, thereby truncating

these receptors in the amino terminal A/B domain in ER $\Delta$ 2 (110), or in different portions of the hormone binding domain in ER $\Delta$ 5, and ER $\Delta$ 7 (95, 97, 110). Exon 4 splicing is in frame, and leads to the deletion of most of the hinge region and a portion of the hormone binding domain (103). Similarly, an in frame skipping of exon 3 deletes the second zinc finger of the DNA binding domain, rendering this form of the receptor incapable of binding to DNA (110).

Of these ER isoforms, ER $\Delta$ 5 has been most explored to date. As with most of the ER isoforms, ER $\Delta$ 5 was first identified in breast tumors. Since it lacks the hormone binding domain (a region also implicated in binding hsp's), ER $\Delta$ 5 was postulated to function as a constitutively active receptor (97). Transient transfection of ER $\Delta$ 5, in conjunction with an ERE-CAT reporter, demonstrated such hormone independent activity (97, 98). Given the focus of such studies on breast cancer, without a critical comparison of the normal tissue, the suggestion that ER splicing is an abnormal event associated with cancer was put forth (92, 101, 108). This was an attractive notion in light of the well documented progression of breast cancers from hormone dependence and tamoxifen sensitivity to hormone independence and tamoxifen resistance, as well as the observation that tumors occasionally exhibit a discordant ER-/PR+ phenotype (as measured by ligand binding assays which would not detect ER $\Delta$ 5) (2). Furthermore, a breast tumor cell line (BT-20), previously classified as ER-, was found to express both ER $\Delta$ 5 mRNA and, importantly, ER $\Delta$ 5 protein (98). This demonstration that an ER mRNA splice isoform is

endogenously translated into a stable protein *in vivo*, was the first indication that ER may in fact be a family of receptor proteins.

Since these initial studies, several groups have demonstrated that ERΔ5 mRNA, as well as several other isoforms, are in fact expressed in both normal breast (reduction mammoplasty specimens) and endometrial tissue (94, 96). Furthermore, although promising during the initial *in vitro* experiments which showed that this receptor isoform could function as an estrogen independent constitutive transactivator (97, 98), a thorough analysis of stably expressed ERΔ5 in MCF-7 cells did not reveal a change in cell growth or malignant potential (99). Furthermore, contrary to the prediction that MCF-7 cells overexpressing ERΔ5 should be estrogen independent and tamoxifen resistant, these cells remained responsive to both estrogen stimulation and tamoxifen inhibition, at levels comparable to that observed in the controls (99). Consequently, these data suggest that ERΔ5 does not play a role in the progression of breast cancer to estrogen independence, and its function in estrogen responsiveness *in vivo* remains unclear.

#### ***Non-DNA binding ER isoform (ERΔ3):***

To date the only reported analyses of tissue distribution of ERΔ3, outside of the data presented in this thesis, are limited to primary breast tumors and two breast cancer cell lines, showing that ERΔ3 is a minor component of total ER mRNA in cancer cells (ERΔ3 representing less than 10% of total ER) (110, 111).

Functional studies of ERΔ3, consisting of a series of *in vitro* translation and transient transfection experiments, demonstrated the dominant negative activity of ERΔ3 on ER function (110). When co-translated *in vitro*, ERΔ3 showed a concentration-dependent inhibition of ER binding to its specific DNA response element (ERE) (110, 111). However, while approximately a 50% reduction in ER binding would be expected from a 1:1 ratio of ERΔ3 to ER if hetero- and homo-dimers were formed with similar efficiency, only 25% reduction was observed; in fact a 4:1 ratio of ERΔ3 to ER was necessary to attain a 53% inhibition of ER binding to ERE (110). Similar results were obtained in transient co-transfection experiments of HeLa cells, in which transfection of ER and ERΔ3 with an ERE-CAT reporter showed ERΔ3 suppression of estradiol (E2) stimulated ER transactivation (110). A 1:1 ratio of ERΔ3 to ER yielded a comparable 30% inhibition of ER transactivation of the ERE-CAT reporter (110). A maximal inhibition of estrogen stimulated gene expression (almost 90%) was observed when a twenty fold excess of ERΔ3 was transfected (110).

These experiments suggested that if expressed at sufficiently high levels relative to full length ER, ERΔ3 may have a profound suppressive effect on estrogen dependent functions; alternatively, its lack of expression may allow for an increased magnitude in estrogen mediated pathways.

As stated, in breast cancer ERΔ3 represents only a minority of the total ER mRNA (111). However, the heterogeneous contribution

of normal mammary epithelium and stroma to the total mRNA extracted from breast tumors, complicate the interpretation of the cellular source of ERΔ3 in these studies, necessitating a corresponding analysis of ERΔ3 expression in normal mammary cells. In addition, an assessment of continual ERΔ3 action *in vivo* is required to conclusively demonstrate its dominant negative function as well as investigate its participation in signal transduction independent of its inhibition of ER (possibly via the AP-1 pathway). Finally, although its stability in *in vitro* translation and transient expression suggests that ERΔ3 mRNA can be translated into protein *in vivo*, the identification of endogenous ERΔ3 protein is necessary to establish its role as a mediator of estrogen action.

By examining the relative contribution of ERΔ3 to ER in normal and cancer breast tissue, this thesis set out to determine the importance of ERΔ3 in breast tumorigenesis. Several possibilities exist with regard to prediction of ERΔ3 function in the mammary gland. The relative, high expression of ERΔ3 in normal breast tissue may provide a means of down regulating estrogen responses, especially during estrogen surges, while the relative loss of ERΔ3 in breast tumor tissue may lead to unchecked estrogen stimulation. Alternatively, a rise in ERΔ3 expression during breast carcinogenesis may facilitate the disabling of the normal differentiation-inducing function of estrogen. Finally, the isoform may represent such a minor component that it would not influence estrogen mediated pathways in either normal or malignant tissue.

To distinguish between these possibilities a comparison of the relative levels of ERΔ3 and ER expression was carried out in breast cancers and cancer cell lines, and normal breast epithelial cells, and fibroblasts, purified from reduction mammoplasty specimens. This comparison, and the subsequent analysis of breast cancer cells stably expressing ERΔ3 mRNA and protein, provided strong support for a hypothesis suggesting that a selective loss of ERΔ3 from normal mammary epithelium is an important early event during oncogenic transformation perhaps underlying the mitogenic actions of estrogen in breast cancer.

## **MATERIALS AND METHODS:**

### ***Separation of epithelial and stromal cells:***

Normal reduction mammoplasty specimens or breast cancer samples were obtained from the Pathology Department, Mount Sinai Medical Center. Epithelial organoids were separated from stroma by clearing the tissue of any obvious fat, mincing and incubating overnight in hyaluronidase/collagenase as described (115). The glandular organoids were collected by filtering through a 400 mesh sieve, thus trapping the epithelial component and allowing the single cell fibroblasts to pass. The fibroblasts were collected and seeded in DMEM supplemented with 10% fetal bovine serum (FBS) (JRH, Lenexa, KS). Some glandular organoids, not purified further and marked "unselected", were collected and seeded in a small volume of mammary epithelial cell growth medium (MEGM; Clonetics, San Diego CA) with 5 ug/ml transferrin and 10 uM isoproterenol, or subsequently used for the isolation of luminal and basal epithelial cells (described below).

For purification of fibroblasts from tumors, tumor tissue was minced and incubated in collagenase for 2 hrs at 37° C. Tumor digests were plated without filtration and the cultures enriched for fibroblasts by differential trypsinization (fibroblasts are the first cells to detach).

### ***Separation of basal and luminal epithelium:***

Basal epithelial cells were positively selected from trypsinized epithelial organoids using a monoclonal antibody to the CALLA antigen (DAKO, Carpinteria CA) and Dynabeads (Dynal, Norway) coated with goat anti-mouse IgG (a 10:1 bead:cell ratio) essentially as described (116). The basal epithelia enriched cells were cultured in MEGM supplemented with 5 ug/ml transferrin and 10 uM isoproterenol, while the CALLA negative fraction, containing luminal cells, was densely seeded in the same medium onto collagen I-coated dishes. After a week in culture, RNAs were extracted and cell purity determined by Northern blot analysis of cytokeratin expression (K8-luminal and K5-basal). Cell preparations with K8 to K5 ratios of 10 to 1 or 1 to 10, were defined as luminal or basal cells, respectively.

***Estrogen receptor RT-PCR:***

Total RNA was extracted using RNazol B reagent (Biotecx Laboratories, Houston, TX), as per manufacturer's recommendations, and 1 ug was reverse-transcribed using Superscript reverse transcriptase (Gibco BRL, Githerburg, MD) and a primer specific to exon 4 (5'-GGAGACATGAGAGCTGCCAAC-3') of ER. This Exon 4 primer and a primer specific to exon 2 (5'-CCGCAAATGCTACGAAGTGG-3') were used to amplify ER-cDNA in a 25 cycle reaction of 1 min. each at 95°, 60° and 72°. PCR products were fractionated on a 2% agarose gel, Southern blotted onto Hybond nylon membrane (Amersham, Arlington Heights, IL), and probed using either a <sup>32</sup>P end-labeled internal exon 4 probe (5'-GAATGTTGAAACACACAAGCGCC-3'), detecting full length ER and ERΔ3 or an exon 3 specific probe (5'-CCGCAAATGCTACGAAGTGG-3'),

detecting full length ER only. Quantitation was performed using the phosphoimager ImageQuant program.

*Immunofluorescent detection of intermediate filament proteins and ER:*

Cells were seeded on sterile, glass coverslips, allowed to attach overnight, washed 2X with PBS, fixed and permeabilized with 2% paraformaldehyde containing 0.1% Triton-X 100 for 5-10 min., at room temperature, and blocked in PBS containing 1% of bovine serum albumin (1% PBSA) for 15 min.. Primary antibody was incubated in 0.2% PBSA either overnight at 4°C (rat anti-ER antibody - H226), or for 1hr at room temperature (mouse anti-smooth muscle actin ( $\alpha$ -SM-actin) antibody (Sigma) and mouse anti-K8 antibody (Jackson Biosciences). Coverslips were washed 3x10 min. with 1% PBSA, and incubated with secondary antibody conjugated either to biotin (goat anti-rat IgG-Biotin, for H226 detection), or rhodamine (goat anti-mouse IgG-rhodamine, for anti- $\alpha$ -SM-actin and anti-K8 detection) for 45 min. at room temperature. For detection of the biotin conjugated antibody, the coverslips were washed 3x10 min. in 1% PBSA, and incubated with strept-avidin conjugated rhodamine for 30 min. at room temperature. After a final wash sequence, coverslips were mounted on slides using Vector-Shield (Vector Biosciences), sealed with clear nailpolish, and stored at 4°C, in the dark until viewing (no more than two days). Microscopy and photography was performed using a Zeiss fluorescent microscope. All secondary antibodies were obtained from Sigma.

***Preparation of ERΔ3 (ERΔ3/pMV7) expression vector:***

A partial ER-cDNA fragment, containing exons 1, 2 and 4, but missing exon 3 (111), (a gift of Dr. R. Miksicek, SUNY, Stony Brook, NY) was used to replace exon 1 - 4 in a similarly digested HEGO vector (a gift of Dr. P. Chambon, Strasbourg, France). The resulting ERΔ3 coding sequence was purified and ligated into a retroviral expression vector containing the neomycin resistance gene, with pMV7 (117) under the MuLV promoter. The "empty" pMV7 plasmid served as a vector control. Both vectors were used to transform DH5 $\alpha$  bacteria and the DNAs purified using Wizard Maxi-Prep kit (Promega, Madison, WI), as per manufacturers recommendation. To prepare ERΔ3-coding retrovirus for infection, ERΔ3/pMV7 DNA was transfected into an amphotropic packaging cell line Ψ-CRIP, selected with G418, the virus was collected, and used for infection.

***Transient transfection of COS cells with the ERΔ3/pMV7 expression vector:***

Transient transfection was performed with 3ug of purified ERΔ3/pMV7 cDNA using Lipofectin, as per the manufacturer's recommendation. Transfected cells, allowed to recover overnight, were attached to glass coverslips for 16 hrs. and stained for expression of ERΔ3 protein using a rat anti-ER antibody, as described above.

***Maintenance of control and ERΔ3 clonal cells:***

MCF-7 cells were maintained in RPMI-1640 medium supplemented with insulin (5 ug/ml), penicillin (50U/ml), streptomycin (50

ug/ml), and 10% FBS. All transfected cells were maintained in selection medium with 500 ug/ml G418. For growth of ERΔ3 clones, 10% charcoal stripped FBS (csFBS) was used, unless otherwise noted.

***Preparation of charcoal stripped FBS:***

1.25gm of activated charcoal (Sigma) and 0.125gm of dextran (Sigma) was added to a 500ml bottle of FBS, incubated at 55°C for 30 min., and centrifuged at 3000 rpm for 20 min.. The resulting single stripped serum was collected into a fresh 500ml flask and the procedure repeated with a second round of charcoal/dextran, but incubated at 37°C for 30 min.. This serum, depleted of steroid hormones, was filter sterilized, and used for growth of ERΔ3 expressing clones.

***Generation of MCF-7 cells expressing ERΔ3  
(Transfection/Infection):***

Three ug of ERΔ3/pMV7 or pMV7 DNA was transfected with Lipofectin into MCF-7 cell, as per manufacturer's recommendation. For retroviral infection (118) 2 ml of growth medium containing the virus and 8ug/ml of polybrene were added to semi-confluent MCF-7 cells, the cells were rocked for 2 hrs at 37°C, the inoculum removed, cells incubated in medium with serum for 48 hrs. and transferred into selection medium containing G418. Infected and transfected cells were maintained in G418-containing medium for 1-2 months prior to clone isolation.

***Immunoprecipitation and western blotting:***

Total cell protein was prepared by 4 freeze/thaw cycles in a high salt lysis buffer (0.4M NaCl, 10% Glycerol, 1mM DTT, 100mM Tris, 10mM EDTA, 50 ug/ml Leupeptin, 50 ug/ml Aprotinin, 10 ug/ml Pepstatin). ER and ERΔ3 were immunoprecipitated with a rabbit anti ER antibody (Zymed, San Francisco, CA), and protein G agarose (Boehringer Mannheim, Indianapolis, IN) from 400 ug of total protein diluted with lysis buffer without NaCl for a final NaCl concentration of 0.2 M. Immunoprecipitated material was resuspended in 50ml of loading buffer, and electrophoresed on an 11.5% SDS/PAGE gel for 8 to 10 hrs at 200 Volts. Protein was transferred onto nitrocellulose membrane (Amersham), blocked overnight with 5% non-fat milk, washed in TBST/1% non-fat milk, western blotted with H226 (0.7mg/ml) overnight at 4°C, and incubated with an HRP-conjugated goat anti-rat secondary antibody (1:10,000 dilution) (Sigma, St. Louis, MO) for 1hr at room temperature. Chemiluminescence (ECL Kit, Amersham) detected bands were quantitated by densitometry.

*Phosphatase treatment of protein extract from pMV7 and ERΔ3 clone:*

Total ER was immunoprecipitated from 2 mg of protein from pMV7pool and ERΔ3 clonal cells, using rabbit polyclonal anti-ER antibody (Zymed). Immunoprecipitated material was split into two equal aliquots, resuspended in 25 ul 1X phosphatase buffer (Boehringer Mannheim, 10X phosphatase buffer: 0.5 M Tris-HCl pH 8.5, 1 mM EDTA), containing a 2X protease cocktail (100 ug/ml Leupeptin, 200 ug/ml Bacitracin, 100 ug/ml Aprotinin, 20 ug/ml

Pepstatin). One aliquot of each of pMV7pool and ERΔ3-3, was treated with 3 Units of calf intestinal phosphatase (CIP) (Boehringer Mannheim), and along with the mock treated aliquots, were incubated for 30 min. at 30°C. The reaction was terminated by the addition of 25 ul of 2X loading buffer and heating to 95°C for 3 min. western blot analysis was performed as described above.

***Expression of pS2:***

pMV7pool and ERΔ3-1, 2, 3, and 4 clonal cells ( $1 \times 10^6$ ) were grown for 3 days in 100mm tissue culture dishes in the presence of FBS, and subsequently treated with either ICI 164,384 ( $1 \times 10^{-7}$ ) or two concentrations of E2 ( $1 \times 10^{-8}$ M and  $1 \times 10^{-10}$ M) for 2 days. pS2 expression was assessed by northern blotting 20 ug of total RNA, as previously described, hybridized with random primed pS2 and GAPDH (as a loading control) cDNA probes. pS2-mRNA level was determined by densitometric analysis.

***Regulation of ER and ERΔ3 proteins:***

pMV7pool and ERΔ3 clonal cells ( $2 \times 10^6$ ) were grown for 2 days in 100mm tissue culture dishes in csFBS, and subsequently either kept in FBS alone or treated with Tamoxifen ( $1 \times 10^{-6}$ ) or E2 ( $1 \times 10^{-8}$  M) for 24 hours. Expression of ER and ERΔ3 protein was assessed in 500ug of total cell lysate by immunoprecipitation and western blotting, as described above, and quantitated by densitometry.

***Saturation density:***

pMV7pool and ERΔ3 clonal cells ( $4 \times 10^6$ ) were plated in 100 mm tissue culture dishes in FBS and  $1 \times 10^{-8}$  M Estradiol (E2) (Sigma). Cells were maintained for four days beyond visual confluence, with a medium change every 2 days, trypsinized and counted. Mean and standard deviation were calculated from four independent experiments.

***Fluorescent labeling of spheres:***

Spheres were collected from a confluent culture of ERΔ3 clone-2 cells, washed with 10ml of PBS, and allowed to settle. 10mM BCECF-AM (2',7' -bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxyethyl ester; Molecular Probes Inc., Eugene, Oregon), a pH indicator used to label live cells, was prepared in DMSO, and diluted at 1:300 in HEPES buffer. Spheres were loaded by incubating for 30 min. at 37°C and visualized using fluorescent confocal microscopy (Zeiss).

***Growth in soft agar:***

A two layer low melt agarose (Seaplaque) system was used to assess anchorage independent growth of pMV7pool and ERΔ3 clonal cells. A 1% lower layer and an 0.4% upper layer of agarose, prepared in DMEM medium supplemented with insulin (5 ug/ml), penicillin (50 U/ml), streptomycin (50 ug/ml), and 10% FBS (+/- E2  $1 \times 10^{-8}$  M or +/- Tamoxifen  $1 \times 10^{-6}$  M) was inoculated into 60mm gridded plates. Cells ( $2 \times 10^3$  cells/ml), distributed in the upper layer, were allowed to grow for 2 weeks and colony formation in the three

conditions was scored. The effect of E2 and Tamoxifen was determined by comparison to cloning efficiency in FBS.

*Chorioallantoic membrane invasion:*

Invasion was assayed as previously described (119). ERΔ3 clones or pMV7pool cells were grown in the presence of selection medium supplemented with 10% FBS and estradiol ( $1 \times 10^{-8}$  M) for 48 hrs.. Cells were trypsinized, counted, allowed to attach overnight in the same medium ( $4 \times 10^6$  cells per 100-mm dish), and labeled with 0.2 mCi/ml of  $^{125}\text{I}\text{UDR}$  for 24 hrs. (specific activity of 0.1 - 0.2 cpm/cell). An artificial air chamber above the CAM of a 10 day old embryo was created, the CAM was allowed to reseal for 22 hrs., and the labeled cells ( $3 \times 10^5$  per CAM) were inoculated onto the CAM. Following a 24 hr. incubation, CAMs were washed with PBS, excised, incubated for 20 min. in trypsin-EDTA (0.05% trypsin, 1mM EDTA), to remove surface attached tumor cells, and rinsed with PBS. The radioactivity remaining in CAMs, after the PBS wash and trypsin-EDTA incubation, is expressed as percent of total radioactivity (associated with CAMs before trypsinization and PBS washes), and represents the proportion of cells that invaded. The invasiveness of ERΔ3 clone-1, -2, -3, -4 and control MCF-7 cells was determined using eight CAMs per cell line, and the statistical significance assessed by the Mann-Whitney U test.

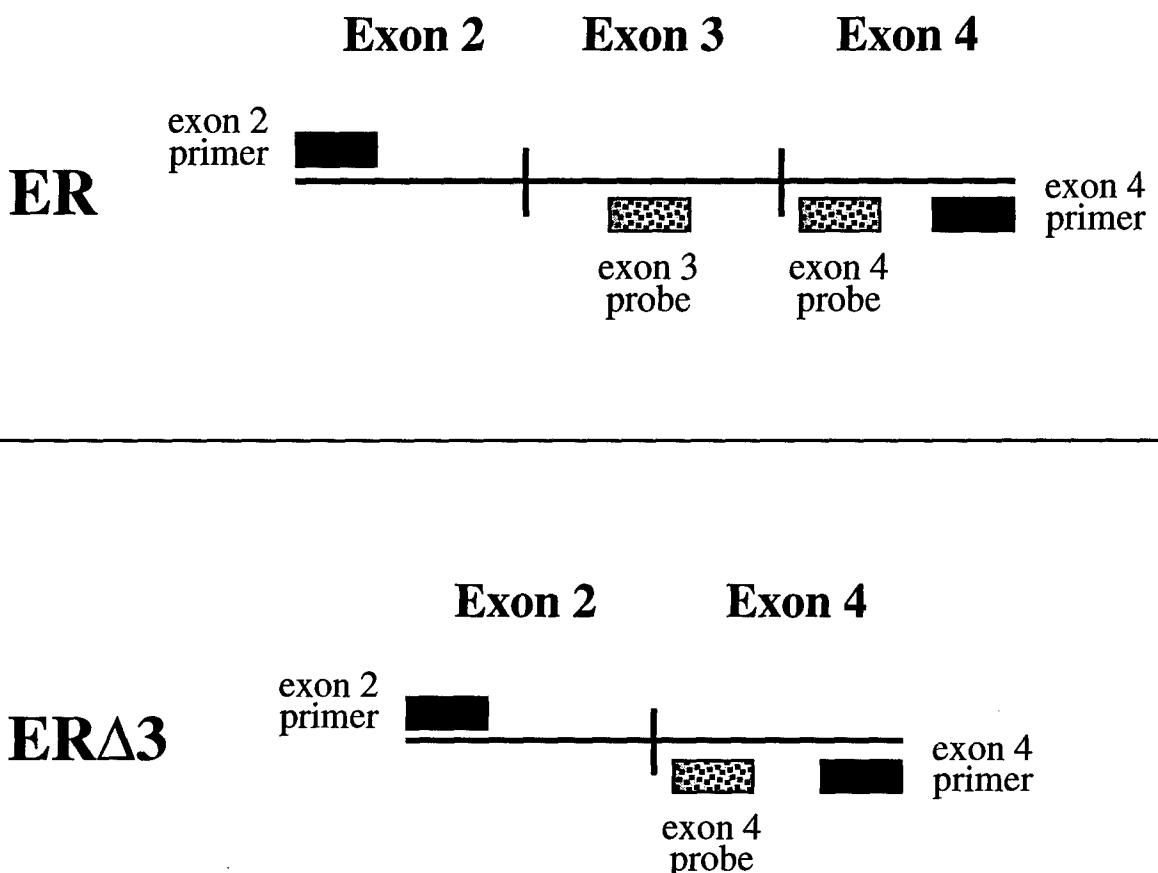
## RESULTS:

As stated in the introduction, several working hypotheses with regard to the role of ERΔ3 in breast cancer were conceivable; a high relative expression of ERΔ3 in normal breast tissue may regulate the magnitude of estrogen responses, particularly during estrogen surges, and a loss of ERΔ3 in breast tumors may result in unchecked estrogen stimulation; alternatively, if ERΔ3 expression increases during breast carcinogenesis it may interfere with the normal differentiation-inducing function of estrogen; finally, this isoform may not affect estrogen action in either normal or malignant tissue, if it represents a very small fraction of total ER. A determination of ERΔ3 to ER ratio in normal versus tumor mammary tissue was necessary in order to discriminate between these possibilities and establish the importance of ERΔ3 in breast cancer.

### *Expression of ER and ERΔ3 in normal breast epithelium and fibroblasts:*

We determined the expression of ERΔ3 and ER in normal isolated mammary cells, breast tumors and breast cancer cell lines. RNAs extracted from these specimens were analyzed using a semi-quantitative RT-PCR assay capable of distinguishing between mRNA encoding the full length and the ERΔ3 form of the receptor. cDNA was prepared using a specific antisense primer complementary to a sequence in exon 4 of ER, and amplified with the same primer and a primer complementary to exon 2 (Fig. 2 shows a schematic of this assay). Amplified products were fractionated, in duplicate, on

agarose gels and transferred onto a nylon membrane. Amplified plasmid DNAs containing either the coding region of ER $\Delta$ 3 or ER were used as size markers in all experiments. In order to determine the specificity of amplification, replicate filters were hybridized with internal probes either within exon 4, to detect both forms of the receptor, or within exon 3 to detect only the full length ER. Filters were exposed to a phosphoimager screen and signal intensity of ER $\Delta$ 3 and ER quantified.

**Figure 2**

**Figure 2*****Schematic representation of RT/PCR Assay.***

Total RNA (1ug) is reverse transcribed using an antisense primer complementary to exon 4 of ER, and the resulting cDNA amplified with the same exon 4 primer and a primer complementary to exon 2 for 25 cycle of 2 minute each at 95°, 60°, 72°. Fractionated products are transferred in duplicate onto a nylon membrane and hybridized with a  $^{32}\text{P}$ -end labeled internal probe to exon 4, to detect both ER and ERΔ3, or exon 3, to detect full length ER only.

Using the RT/PCR assay described above, we determined the estrogen receptor composition in normal breast cells. Since the cellular composition of a total homogenate of normal reduction mammoplasty specimens, rich in adipose stroma with intervening epithelial glands, is not comparable to that of primary breast cancers, predominantly composed of epithelial tumor cells, a cell isolation procedure was used to directly compare ER $\Delta$ 3 to ER ratios of normal epithelial cells to the primary breast cancers and cancer cell lines. Normal mammary basal/myoepithelial cells, luminal epithelial cells and fibroblasts were purified from reduction mammoplasty specimens using established methods for cell separation (115, 116). Briefly, surgical reduction mammoplasty specimens were cleared of fat tissue, minced and digested with collagenase and hyaluronidase to disrupt connective tissue. The released epithelial organoids were filtered through a defined diameter porous sieve, thus trapping the glandular/organoid structures and allowing passage of single cell fibroblasts and other cells. The fibroblast fraction, containing both fibroblasts and myofibroblasts, was subsequently collected and seeded in FBS supplemented DMEM. Myofibroblasts were identified by immunofluorescent detection of their contractile filaments, using an anti-smooth muscle actin antibody (Fig. 3).

**Figure 3**



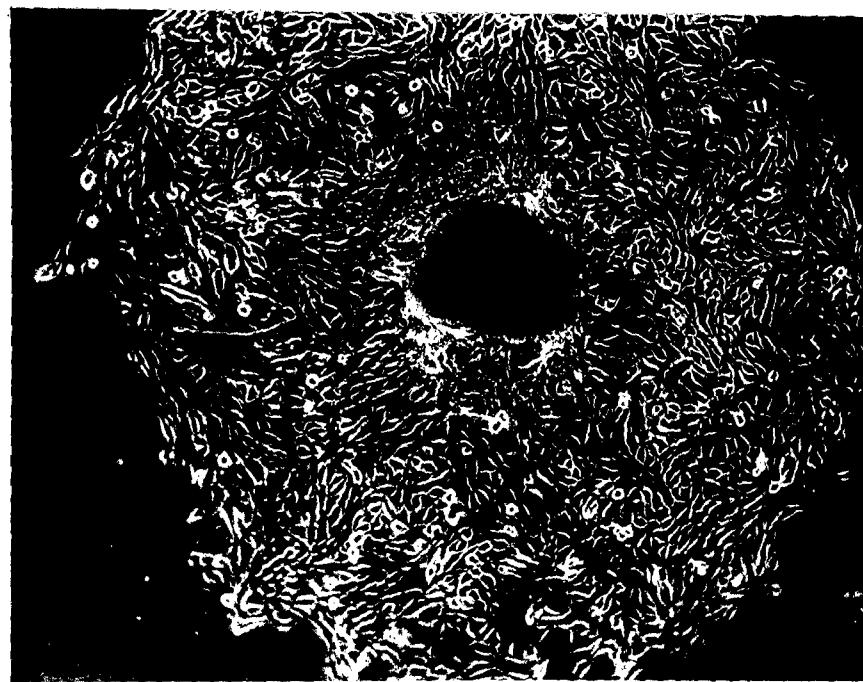
**Figure 3*****Immunofluorescent detection of  $\alpha$ -smooth muscle actin expression in mammary myofibroblasts.***

Isolated fibroblasts from reduction mammoplasty specimens were plated onto glass coverslips, fixed and permeabilized with 3% paraformaldehyde containing 0.1% triton-X 100, and incubated with a mouse anti- $\alpha$ SM-actin antibody for 1 hr. at room temperature. Cells were washed with 1% PBSA, incubated for 45 min. at room temperature with goat anti-mouse IgG conjugated to rhodamine, and visualized using a Zeiss fluorescent microscope. Image shows staining of the  $\alpha$ SM-actin organized into stress fibers in mammary myofibroblasts.

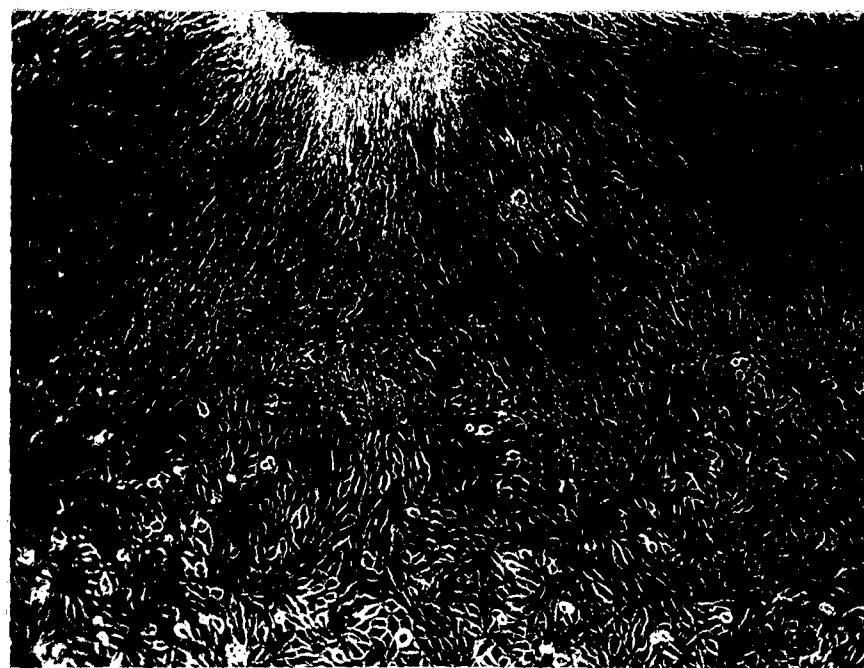
Epithelial organoids, plated onto culture dishes, settled as dense spheres of cells which after several days in culture were encircled by an epithelial outgrowth (Fig. 4, a and b). Basal cells were seen on the outside boarders of the migrating cells, and luminal cells, in the area immediately surrounding the sphere. These two distinct cell populations are easily distinguished by their morphology. As shown in a higher magnification of an organoid (Fig. 4c), the luminal epithelial cells are smaller and denser with few cytoplasmic projections, as compared to the surrounding basal cells.

**Figure 4**

*a.*

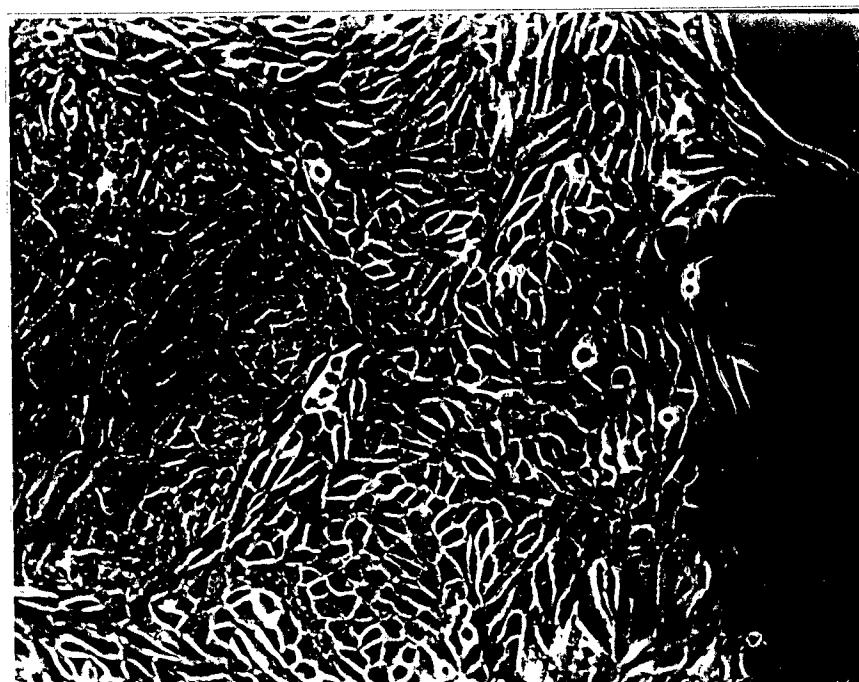


*b.*



**Figure 4**

*c.*



**Figure 4*****Phase Contrast of purified epithelial organoids in culture.***

Reduction mammoplasty specimens were minced and digested to disrupt connective tissue, and epithelial organoids were collected as described. *a.* An unfolding organoid is seen in the center as a round, dense sphere of cells, immediately surrounded by a small outgrowth of dense luminal cells. Elongated basal cells are the majority of cells seen around the glandular structure. *b.* A much larger organoid than shown in *a* is seen at the top edge of the image. Luminal cells immediately next to the organoid are readily observed as small, dense cells containing granular material in their cytoplasm, and are surrounded by the morphologically distinct basal epithelial cells. (Note that images *a* and *b* are of the same magnification). *c.* A higher magnification view of *b* demonstrates the restricted border in the pattern of outgrowth of these two cell types. Their differing morphologies are easily evident with luminal cells on the left and basal cells on the right side of the image.

In addition to their different morphology, these two cell types can also be distinguished by their intermediate filament, cytokeratin, expression (20). An example of the specificity of such identifying markers is demonstrated in Figure 5. A glandular organoid containing both the luminal and basal cells was examined by immunofluorescence using an antibody to cytokeratin 8 (K8). Luminal cells, which express high levels of K8 (Fig. 5a) show bright cytoplasmic staining. Phase contrast of the same field (Fig. 5b) shows the luminal cells in the center surrounded by basal epithelial cells at the periphery, negative for K8 expression. RNA was prepared from these mixed epithelial cell cultures and subsequently used in the RT/PCR analysis to determine the ERA3 to ER ratio.

**Figure 5***a.**b.*

**Figure 5*****Immunofluorescent detection of cytokeratin 8 in luminal epithelial cells.***

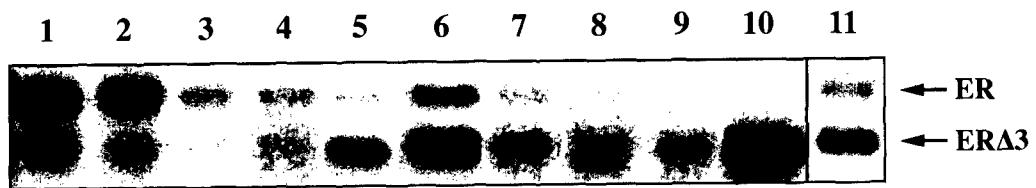
Epithelial organoids were isolated from reduction mammoplasty tissues (as described in materials and methods), plated onto glass coverslips, fixed, permeabilized, and stained with mouse anti-cytokeratin 8 antibody. Goat anti-mouse secondary antibody conjugated to rhodamine was used for detection, and visualized with a Zeiss fluorescent microscope. *a.* Luminal cells are brightly stained with the anti-K8 antibody *b.* Phase contrast of the same image shows the luminal cells in the center surrounded by basal cells at the periphery, negative for K8 expression.

For preparation of pure populations of luminal and basal cells, before plating, the organoids were trypsinized into a single cell suspension, and reacted with a primary antibody specific for the CALLA antigen present on basal cells. Using magnetic beads conjugated to a secondary antibody, cells with anti-CALLA antibody were separated. This purification yields a CALLA-positive fraction containing the basal cells, and a CALLA-negative fraction enriched in luminal epithelium. The two fractions were either cultured under conditions supporting basal (plated on tissue culture dishes in MEGM) or luminal (densely plated onto collagen I coated dishes in MEGM) cell growth. Total RNA was extracted, and the cell types confirmed using northern blot analysis of cytokeratin expression (K5 for basal cells and K8 for luminal cells). Only K8/K5 ratios of 10:1 were deemed as pure luminal cell preparations.

The analysis of normal epithelial cells, isolated from 10 reduction mammoplasties, and one, non-transformed, immortalized myoepithelial mammary cell line (Hs 578Bst) yielded a median ratio of ER $\Delta$ 3 to ER of 3.4 (range 0.4 to 9.8) (Fig. 6; Fig. 7 group 3). Figure 6 shows the RT/PCR products, fractionated on an agarose gel, transferred onto a nylon membrane and hybridized with the exon 4 probe to detect both the full length and ER $\Delta$ 3 forms of the receptor. Figure 7 shows a composite of the ER $\Delta$ 3 to ER ratios from several such RT/PCR analyses (note the logarithmic scale of the y-axis).

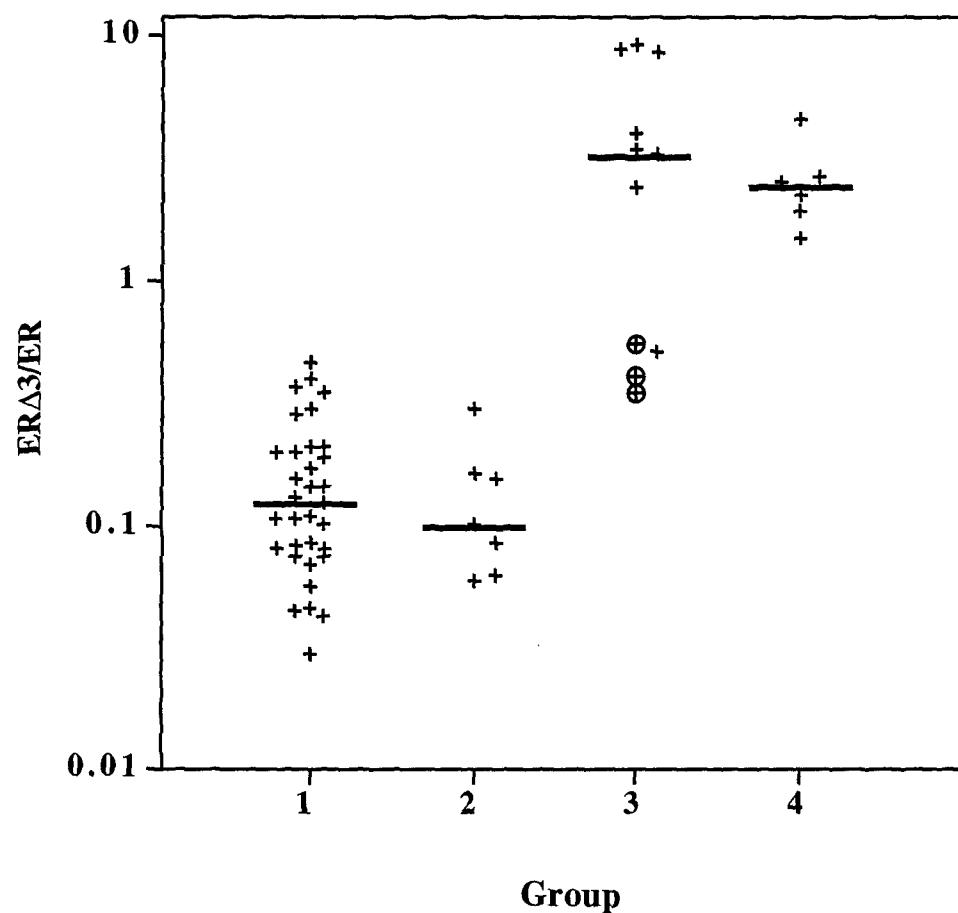
Of the 10 samples represented, three were purified luminal epithelial cell preparations (Fig. 6, lanes 1-3; Fig 7, group 3, encircled

crosses). Their ERΔ3 to ER ratio was determined to be 0.42 (range 0.36 to 0.53). The analysis of basal cells and unselected epithelial cells, most of which contained predominantly basal cells, indicated that the median ERΔ3 to ER ratio was even greater (median 4.0; range 0.55-9.8) (Fig. 6, lanes 4-10; and Fig. 7, group 3).

**Figure 6**

**Figure 6*****Analysis of ER and ERΔ3 mRNA expression in purified normal breast epithelial cells.***

Southern blot of cDNAs obtained by RT-PCR of mRNA of epithelial cells from 10 reduction mammoplasties (lanes 1-10) and Hs 578Bst, a normal, immortalized myoepithelial cell line (lane 11) detected with exon 4 probe (recognizing both ER and ERΔ3). Lanes 1-3 represent purified luminal epithelial cell preparations. Lanes 4-10 represent basal cells and unselected epithelial cells (predominantly containing basal cells).

**Figure 7**

**Figure 7**

*Composite scattergram of the ERΔ3 to ER ratio from RT-PCR analyses of normal mammary cells, breast tumors and breast cancer cell lines.*

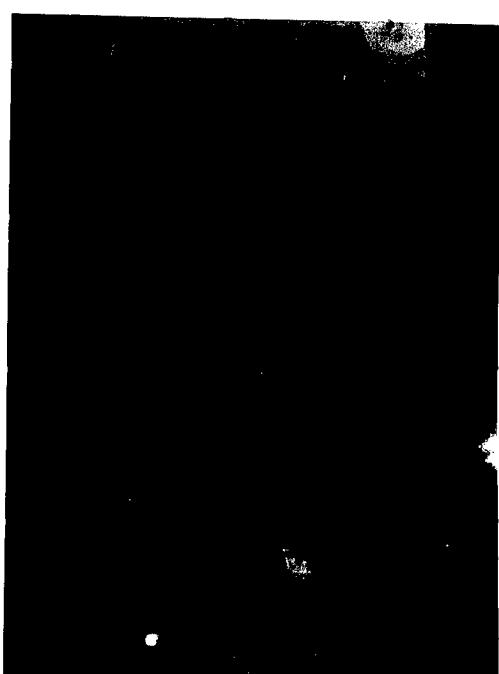
ERΔ3 and ER RNA ratios in breast cancers (group 1), breast cancer cell lines (group 2), normal luminal epithelium (group 3) (the pure luminal epithelium, n=3, indicated by circled crosses), and stromal fibroblasts (group 4) (isolated from normal (n=4) and cancer (n=2) breast tissue). Each cross in the scattergram represents the scanned relative intensity of ERΔ3 and ER bands produced by Southern blotting of cDNAs generated by RT-PCR of RNA extracted from an individual tissue or cell sample. The medians of ERΔ3 to ER ratios shown by a horizontal line for each group are as follows: 0.12 for breast cancers (group 1), 0.1 for breast cancer cell lines (group 2), 3.4 for normal epithelial cells (group 3), and 2.4 for fibroblasts (group 4). Note the logarithmic scale of the Y-axis.

Finally, mammary fibroblasts, from reduction mammoplasty specimens and tumors, were assayed for the presence of estrogen receptors. Given the contradictory published data suggesting that human mammary fibroblasts are ER- but responsive to estrogen (13), an analysis of both ER protein and mRNA expression was performed. Isolated mammary fibroblasts were plated onto coverslips, and immunostained using a well characterized estrogen receptor antibody (H226) recognizing an epitope in the amino terminus of ER. Properly localized nuclear ER protein was detected by indirect immunofluorescence using a biotinylated anti-rat secondary antibody bound to a streptavidin rhodamine (Fig. 8a), while mammary fibroblasts incubated with a non-specific IgG primary antibody show no detectable fluorescence (Fig. 8b). ER was detected in the nucleus of ER+ MCF-7 cells while antibody to K8, showing specific cytoplasmic stain, had no nuclear fluorescence (Fig. 8d and e respectively). This data demonstrates that, although expressed at a low level, as compared with MCF-7 cells, ER protein can be detected in mammary fibroblasts using sensitive methodology, and suggests that, as has been observed in the rodent system (13), the effect of estrogen on these cells is most likely mediated via this ER protein.

**Figure 8***a.*

Figure 8

*b.*



*c.*

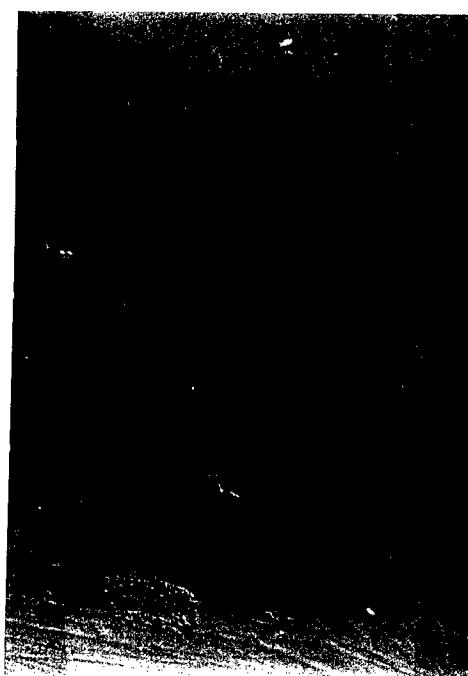
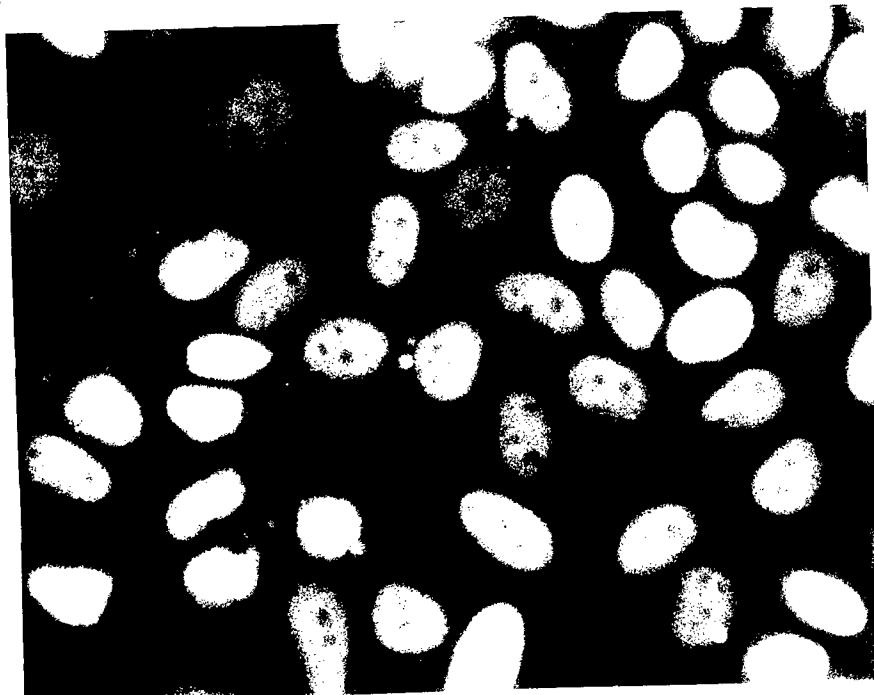


Figure 8

*d.*



*e.*



**Figure 8*****Immunofluorescent detection of ER protein in isolated mammary fibroblasts.***

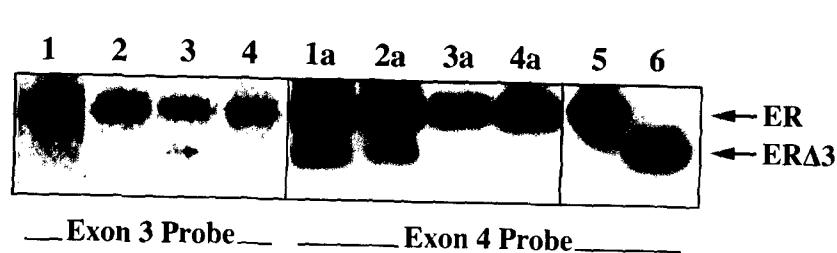
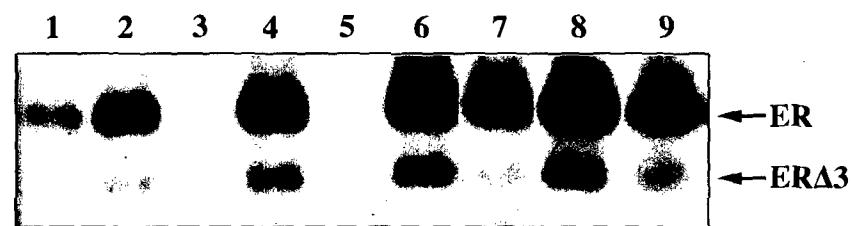
Isolated fibroblasts from reduction mammoplasty specimens (a-c) and MCF-7 cells (d and e) were plated onto glass coverslips, fixed and permeabilized as described in the materials and methods. Primary mammary fibroblasts were incubated with an anti-ER antibody (H226) overnight at 4°C (a) or with non-specific IgG (b). MCF-7 cells were incubated with H226 (d) or anti-K8 (e). For immunodetection a biotin conjugated secondary antibody was followed by streptavidin-rhodamine. *a.* ER specific nuclear staining seen in normal mammary fibroblasts detected with H226 antibody. *b.* Negative control, incubation of normal mammary fibroblasts with a non-specific IgG shows low background fluorescence. *c.* Nomarski optics of the same field as seen in b. *d.* Positive control, ER specific nuclear staining in MCF-7 cells detected with H226 antibody. *e.* MCF-7 cells stained with an anti-K8 antibody show specific cytoplasmic K8, with no detectable nuclear fluorescence.

Since the immunofluorescent detection does not discriminate between ER and ER $\Delta$ 3, the specific expression of these two ER isoforms was determined using the RT/PCR assay described above. Interestingly, breast fibroblasts, like the normal epithelial cells, were found to contain a high ER $\Delta$ 3 to ER ratio (median 2.4, range 1.5 to 4.5), regardless of their source (reduction mammoplasty, n=4 or breast cancer, n=2) (Fig. 7, group 4). The presence of high relative levels of ER $\Delta$ 3 mRNA in mammary fibroblasts suggests that a significant proportion of the detected estrogen receptor protein in these cells may be ER $\Delta$ 3.

***Expression of ER and ER $\Delta$ 3 in breast cancers and cancer cell lines:***

An analysis of 33 breast tumor RNA revealed that the median ratio of ER $\Delta$ 3 to ER expression in these tumors was 0.12 (range 0.03 to 0.47). Figure 9a shows the result of RT/PCR on RNA from four tumors hybridized with exon 3 probe (lanes 1-4), detecting only the full length ER, and exon 4 probe (lanes 1a-4a), detecting both ER and ER $\Delta$ 3. Lanes 5 and 6 show the amplified plasmids, ER $\Delta$ 3 and ER also hybridized with the exon 4 probe. The relative amounts shown are representative of the range of ER $\Delta$ 3 to ER expression in the tumor population (Fig. 7, group 1 shows analysis of all tumors). A similar analysis of 8 breast cancer cell lines, (6 of which were ER positive), and 1 ER positive endometrial cancer cell line produced a median ratio of ER $\Delta$ 3 to ER of 0.1 (range 0.06 to 0.3, Fig. 9b, and Fig. 7, group 2). Therefore pure populations of cancer cells maintained in culture for prolonged period of times had ratios similar to the relative levels

of ERΔ3 in primary breast tumors. These results, consistent with previous reports, show that ERΔ3 in both primary breast cancer and cancer cell lines represents only 10% of the total estrogen receptor mRNA expressed in these cells (110, 111), an ERΔ3 to ER ratio (0.1) almost 30-fold less than that observed in normal mammary epithelium (3.4) (Fig. 7, groups 1, 2, and 3)

**Figure 9***a.**b.*

**Figure 9*****Analysis of ERΔ3 and ER mRNA expression in breast tumors and breast cancer cell lines.***

*a.* Southern blots of 4 cDNAs, obtained by RT-PCR of mRNA of breast cancers, probed with either an exon 4 probe (to detect both ER and ERΔ3), lanes 1a-4a, or exon 3 probe (to detect only full length ER), lanes 1-4. The examples shown represent the range of ERΔ3 to ER ratios found in breast cancers; they are 0.25, 0.1, 0.08, 0.04 for lanes 1a, 2a, 3a, and 4a, respectively. Amplified ER and ERΔ3 plasmid DNAs probed with exon 4 probe are shown in lanes 5 and 6. *b.* Detection of ERΔ3 and ER with exon 4 probe only. Southern blot of ER positive breast cancer cell lines: lane 1, BT 474; 2, MDAMB175vii; 4, MDAMB361; 6, MDAMB134vi; 7, T47D; 8, MCF-7. An ER positive endometrial cancer cell line, Ishikawa, lane 9. ER negative breast cancer cell lines: lane 3, MDAMB231; 5, MDAMB461.

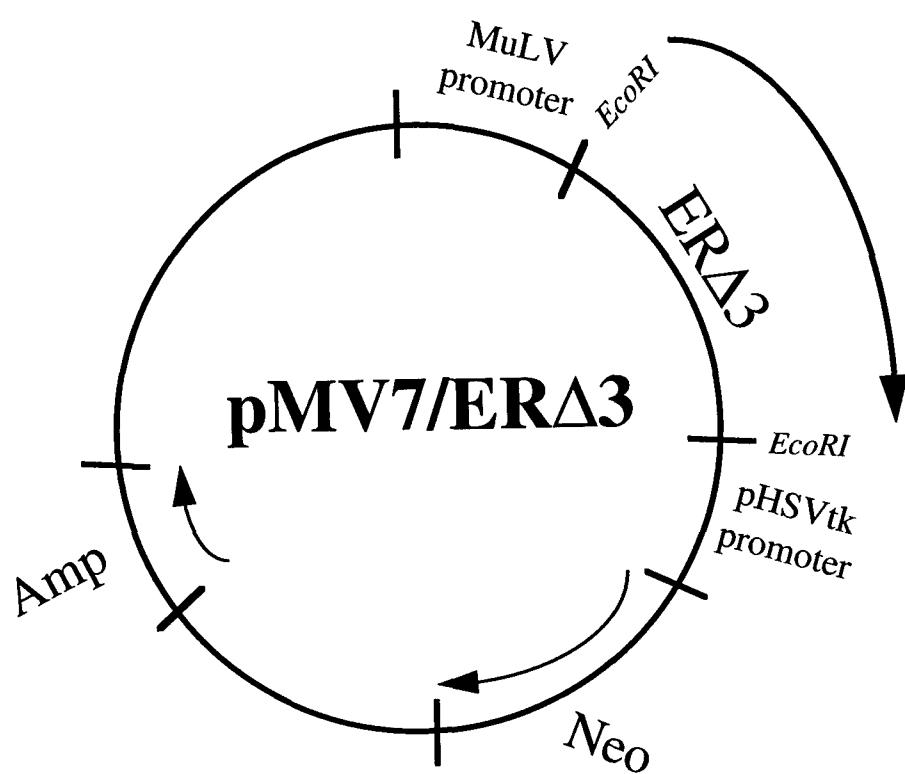
In order to determine whether ER $\Delta$ 3 to ER ratios, were even further decreased with breast cancer progression, the assayed patient population was stratified according to their menopausal status (cancers in premenopausal women are commonly more aggressive) or tumor grade (defined by tumor size, <1.5 cm or > 1.5 cm, or presence or absence of lymph node involvement). This analysis did not identify a significant difference in the ER $\Delta$ 3 to ER ratios between the groups of patients. The exception was a small group of tumors (6/33) with ER levels lower than 5 fmole/mg, as determined by routine ligand binding. These tumors, clinically deemed ER negative, had a median ratio (0.05) significantly different ( $p<0.001$ ) from the median ratio (0.12) of all tumors. The observations that small tumors and tumors that have not spread to lymph nodes, have ratios of ER $\Delta$ 3 to ER comparable to that observed in more advanced tumors, suggests that the loss of ER $\Delta$ 3 may be an early event in carcinogenesis. However, the finding of significantly lower ratios in tumors with a low overall concentration of estrogen receptor, considered more aggressive cancers (2), hints that a further drop of ER $\Delta$ 3 may be associated with disease progression.

These results demonstrated that normal mammary epithelium contains a significantly higher ratio (3.4) of ER $\Delta$ 3 to ER than either breast tumors or cell lines (0.1;  $p<0.001$ ), indicating that a relative loss of ER $\Delta$ 3 occurs with oncogenic transformation. Furthermore data showing that normal luminal epithelium contains a ratio of ER $\Delta$ 3 to ER still almost 4 fold greater (0.42) than the median ratio in primary cancer tissue or cancer cell lines is important in light of findings that

a large proportion of breast cancers express cytokeratin 8 and are believed to have originated from luminal cells (20). In addition, the comparable ratio of ERΔ3 to ER in fibroblasts of both normal and tumor breast, underscore the specificity of the carcinogenic event that leads to the loss of ERΔ3 from tumor epithelium. Given the heterogeneous involvement of stroma in different cancer specimens, such fibroblasts, containing a high ERΔ3 to ER ratio, may contribute to the overall ERΔ3 level in the analysis of some breast tumors. Taken together, these data demonstrate that the ratio of ERΔ3 to full length ER is substantially reduced in breast cancer (30-fold), and suggest that a loss of this isoform is associated with malignant transformation.

***Transfection and isolation of MCF-7 cells expressing ERΔ3; characterization of the native and transgenic protein.***

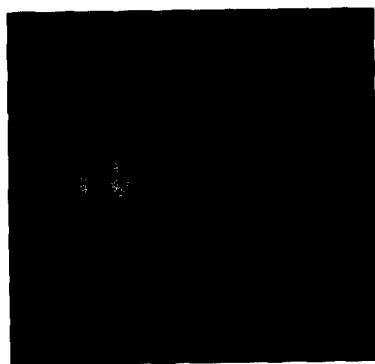
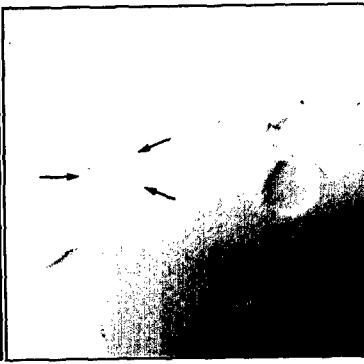
The above findings suggested that restoring ERΔ3 in cancer cells to normal relative levels may result in an attenuation of their transformed phenotype. To test this, ERΔ3-cDNA was constructed by directionally ligating a restriction enzyme digested, gel purified partial ER cDNA spanning exons 1-4 but missing exon 3, (gift of R. Miksicek et al.), into a similarly digested and purified HEGO vector containing partial sequences of exon 1 and exons 4 through 8 of full length ER cDNA (gift of P. Chambon et al.). The resulting ERΔ3 coding sequence was purified and subcloned into the retroviral expression vector, pMV7, containing a neomycin resistance gene (117). This final mammalian expression vector, with ERΔ3 under the control of a MuLV promoter, was used for all subsequent experiments (Fig. 10).

**Figure 10**

**Figure 10*****pMV7-ERΔ3 expression vector.***

ERΔ3 cDNA under the control of an MuLV promoter in the pMV7 retroviral expression vector containing a neomycin resistance gene for G418 selection.

To confirm the ability of this ERΔ3/pMV7 construct to express protein, ER negative COS cells, were transiently transfected and assayed for ERΔ3 expression by fluorescent immunocytochemistry with the anti-ER antibody, H226. Nuclear staining was observed in some cells (Fig. 11a), demonstrating that transfected cells both express and properly localize ERΔ3. In addition, the lack of staining in most cells in the field (Fig 11b), established that the H226 antibody recognizes the ERΔ3 protein.

**Figure 11****a.****b.**

**Figure 11*****Detection of transfected ERΔ3 protein in COS cells.***

COS cells transiently transfected with ERΔ3/pMV7 vector using Lipofectin, were plated on cover slips 16 hrs. later, allowed to attach, fixed with 3% paraformaldehyde and incubated overnight at 4°C with H226 antibody (35ug/ml). Biotin-coupled anti rat IgG secondary antibody and rhodamine conjugated strep-avidin were used for protein visualization. *a.* Immunofluorescent detection of ERΔ3 in nuclei of three COS cells. *b.* Nomarski optic view of the same field.

Prior to stably transfecting MCF-7 cells, sensitivity of the cells to the selection agent geneticin (G418) was tested and established to be 500ug/ml. Lipofection was used to stably transfect cells with either ERΔ3/pMV7 or an "empty" pMV7 vector control DNA. Additional clones were generated by infection. The same retroviral ERΔ3/pMV7 and "empty" pMV7 vectors were transfected, using  $\text{Ca}^{++}$  phosphate, into an amphotropic viral packaging cell line ( $\Psi$ -CRIP cells), virus-containing conditioned medium was collected and subsequently used for stable infection of MCF-7 cells.

Stable clonal lines of MCF-7 cells transfected, or infected, with either the ERΔ3 coding constructs, or the pMV7 vector alone, as a negative control, were selected. Twenty-two ERΔ3/pMV7 transfected and 54 infected clones were isolated, in addition to 10 pMV7 transfected clones and a pMV7 infected pool of cells. Twenty of the twenty two clones selected from the initial transfection survived, but only three were found to express ERΔ3 mRNA; and two of these expressed ERΔ3 protein. The infected clones showed a 55% survival rate (24/54), and subsequent analysis showed that 13 of the 16 clones characterized, efficiently expressed ERΔ3 protein. In contrast, all 10 pMV7 control clones and the pMV7-pool, were successfully selected.

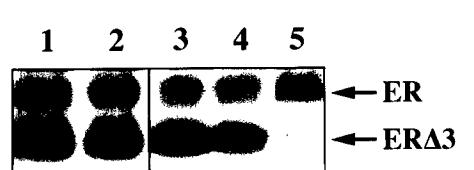
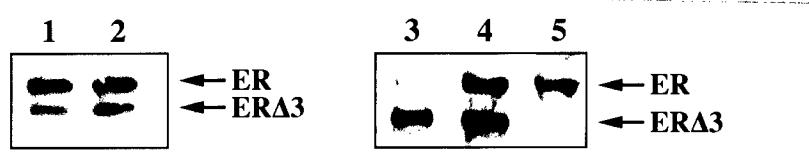
The analysis of ERΔ3 mRNA expression, as determined by RT/PCR, is shown in a subset of clones chosen for subsequent studies (Fig. 12a. lanes 1-4). As expected, control pMV7 cells expressed predominantly full length ER-mRNA and a small amount of ERΔ3,

similar to that observed in the parental MCF-7 cell line (Fig. 9b, lane 8). In contrast, all four ERΔ3/pMV7 clones had a predominance of ERΔ3-mRNA (Fig. 12a), indicating that the transgene mRNA was efficiently expressed in these cells.

In order to identify both endogenous and transgene ERΔ3 protein, and differentiate it from the full length ER, attempts were made to generate specific ERΔ3 antibodies. Since ERΔ3, missing 34 amino acids of the DNA binding domain (encoded by exon 3), is otherwise identical to ER, the only unique sequence of the ERΔ3 protein, the exon 2/exon 4 splice junction, was used to synthesize two peptides. Spanning the junction, one contained a 1:1:1 mixture of 7, 8, and 9 amino acid long peptides, the minimum antigenic length; and the second longer peptide (22 amino acids), had residues homologous to both exons 2 and 4, possibly retaining the conformation of the ERΔ3 splice junction. The peptides were conjugated to carrier proteins, KLH and ovalbumin respectively, and used to immunize both rabbits and mice. This attempt to produce both polyclonal and monoclonal ERΔ3 specific antibodies was unsuccessful, as tested by both western blotting and immunocytochemistry, not entirely surprising given the low antigenicity of the amino acids in the immediate location of the splice junction. Therefore, the ERΔ3 protein was identified on the basis of its reactivity with two antibodies recognizing different N-terminal epitopes of ER, its faster mobility than ER on SDS-PAGE, and the correlation of its expression with that of the ERΔ3-mRNA.

Protein extracts of the individual clones (400ug), shown in figure 12b, were subjected to immunoprecipitation with a polyclonal rabbit anti-ER antibody, and followed by western blotting with the H226 antibody, recognizing the amino-terminus of ER. In addition to the 65 kDa band, representing the full length ER protein, all ERΔ3 clones also contained a prominent 61 kDa band, which corresponded to the predicted molecular weight of the ERΔ3 protein (Fig. 12b). Expression of the ERΔ3 protein in these clones ranged from 30% to 70% of total ER (Fig. 12b, lanes 1-4), a relative ratio of ERΔ3 to ER comparable to that observed in the normal mammary epithelium (range 0.4 to 9.8). In parental MCF-7 extracts, a faint band (~5% of the total ER), co-migrating with the ERΔ3 form, could be detected only when excess protein was loaded onto the gel (Fig. 13, lane 2). This, and the correspondence between the low intensity of the ERΔ3-mRNA band and the 61 kDa protein band (Fig. 9b, lane 8; Fig. 12a, lane 5 and Fig. 13, lane 2, respectively) suggest that both pMV7-carrying and the parental MCF-7 cells produce small amounts of the ERΔ3 mRNA and protein. The identity of the 61 kDa band as ERΔ3, and not as the underphosphorylated form of full length ER, was further confirmed by dephosphorylation of immunoprecipitated estrogen receptors from 1 mg of each, pMV7 and ERΔ3 cell protein with calf intestinal phosphatase (CIP), in the presence of excess protease inhibitors. Analysis of products by western blotting showed that without CIP, ER from both pMV7 and ERΔ3 cells produced a co-migrating doublet of bands, the upper corresponding to full length ER and the lower to ERΔ3 protein (Fig. 13, lanes 2 and 3). CIP treatment shifted the migration coefficient of both bands in the vector control

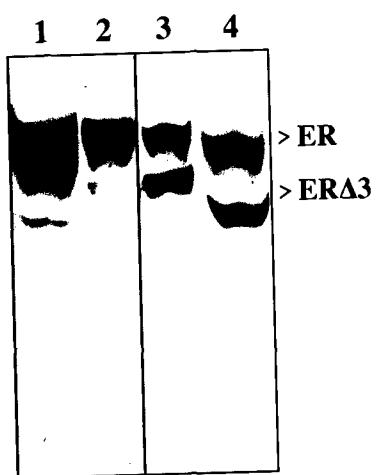
cells as well as the ERΔ3 clone to new positions, once more as a co-migrating doublet (Fig. 13, lane 1 and 4). No lower bands or smear were detected, indicating that proteolysis during the CIP incubation was effectively blocked by the protease-inhibitors cocktail added to the reaction mixtures. Co-migration of the lower molecular weight protein from pMV7 cells with that of the ERΔ3 protein, both before and after de-phosphorylation, strongly suggests the presence of native endogenously produced ERΔ3 protein.

**Figure 12***a.**b.*

**Figure 12**

*Characterization of mRNA and protein from clonal cell lines obtained from stable transfection or infection of MCF-7 cells with ERΔ3/pMV7 or pMV7 alone.*

*a.* Southern blot analysis of cDNA obtained by RT/PCR of mRNA from MCF-7 cells transfected (lanes 1 and 2, ERΔ3-1 and ERΔ3-2) or infected (lanes 3 and 4, ERΔ3-3 and ERΔ3-4) with ERΔ3/pMV7 plasmid DNA (probed with exon 4 probe). Lane 5 represents mRNA from MCF-7 cells infected with pMV7 vector alone (pMV7-pool). *b.* Western blot analysis of the above clones. Total ER immunoprecipitated from 400ug of protein extract. Left panel: experiment 1, lanes 1 and 2, clones ERΔ3-1 and ERΔ3-2, electrophoresis 8 hrs. Right panel: experiment 2, lanes 3 and 4, clones ERΔ3-3 and ERΔ3-4; lane 5, pMV7-pool control electrophoresis 10 hrs. Arrows indicate the 65 kDa ER and 61 kDa ERΔ3.

**Figure 13**

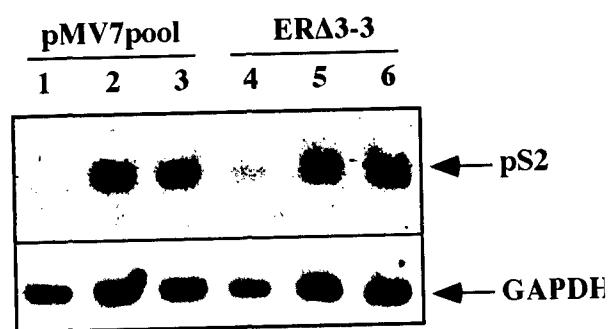
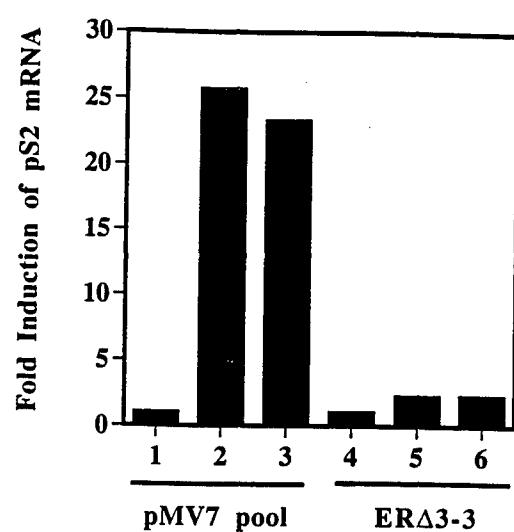
**Figure 13***Identification of the native 61 kDa protein as ERΔ3 on the basis of its dephosphorylation pattern.*

Two equal aliquots of ER immunoprecipitated with rabbit polyclonal anti-ER antibody from 2 mg of protein extracts of pMV7pool or ERΔ3-3 clone grown in medium with FBS (to enhance the ERΔ3 to ER ratio) were resuspended in 25  $\mu$ l of phosphatase buffer with protease inhibitors (100  $\mu$ g/ml Leupeptin, 100  $\mu$ g/ml Aprotinin, 20  $\mu$ g/ml Pepstatin) and incubated for 30 min at 30°C with 3 units (or without, controls) of calf intestinal phosphatase. The products were analyzed by western blotting using the H226 antibody. The amount of protein loaded per lane was 2.5 times more than in Figs. 3 or 6. Lanes 1 and 2, pMV7pool: lane 1, CIP treatment: lane 2, buffer control; lanes 3 and 4, ERΔ3-3, lane 3 buffer control, lane 4, CIP treatment. The dephosphorylated shifted doublets of ER and ERΔ3 are indicated.

***ERΔ3 expression suppresses estrogen stimulated gene expression:***

ERΔ3 has been shown to interfere with ER binding to its specific DNA response element in *in vitro* gel shift experiments, as well as with estrogen induced transcription of an ERE-CAT reporter in transient transfection of COS cells *in vivo* (110, 111). These studies suggested that ERΔ3 functions as a dominant negative receptor to inhibit ER regulation of gene expression through its cognate DNA response element (110). In order to determine whether the ERΔ3 expressed in MCF-7 cells can interfere with estrogen induction of an endogenous gene, the expression of pS2, a gene with several imperfect ERE's in its promoter, was assessed. pMV7 control and ERΔ3 clone cells were incubated either with the pure anti-estrogen, ICI 164,384 ( $1 \times 10^{-7}$  M), to establish the baseline of pS2 expression, or with E2 ( $1 \times 10^{-8}$  M and  $1 \times 10^{-10}$  M). Total RNA was prepared and analyzed by Northern blot to determine pS2 expression. (GAPDH mRNA was used as a loading control.) While E2 treatment of controls induced a 25 fold increase in pS2-mRNA (compare lane 1 with lanes 2 and 3 in Fig. 14a and Fig. 14b), in ERΔ3-expressing cells pS2-mRNA was stimulated merely 2 fold (compare lane 4 with lanes 5 and 6 in Fig. 14a and Fig. 14b). In all additional ERΔ3 clones tested (a total of 4), E2 induction of pS2-mRNA ranged from only 3-9% of that observed in the pMV7 control cells. These results confirm that ERΔ3 interferes with ER regulated gene expression *in vivo*. In addition, since the relative level of ERΔ3 in these clones is much lower than previously shown to be required for efficient dominant negative activity (ERΔ3 to ER ratio of 4:1 was used to achieve a 53% inhibition

of ER binding to an ERE in gel shifts *in vitro* and 20:1 for a 90% inhibition of estrogen induction of the ERE-CAT reporter in COS cells *in vivo*), it is likely that in addition to its inhibitory action on ER transactivation of ERE dependent genes, ERΔ3 may also exert an effect *in vivo* through an as yet unidentified mechanism.

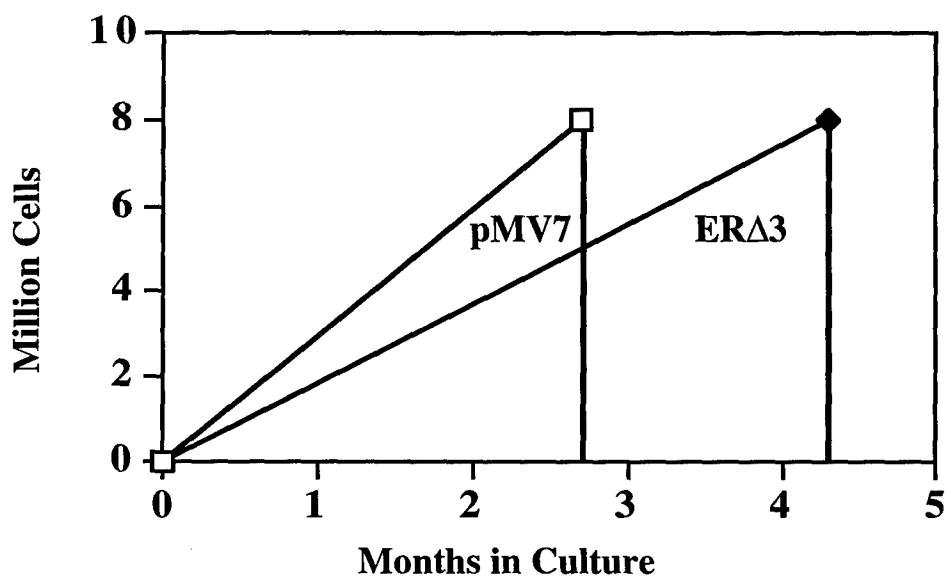
**Figure 14***a.**b.*

**Figure 14*****Effect of ERΔ3 expression on estrogen regulation of pS2-mRNA.***

$1 \times 10^6$  pMV7pool and ERΔ3-3 clonal cells were plated in 100mm tissue culture dishes in the presence of FBS for 3 days and treated for 48 hrs. either with the pure anti-estrogen ICI 164,384 ( $1 \times 10^{-7}$  M), to establish the baseline of pS2 expression , or E2 ( $1 \times 10^{-8}$  M and  $1 \times 10^{-10}$  M). pS2 expression was determined by Northern blot analysis of 20 ug of total RNA (Hybridization with a GAPDH-cDNA probe was used as a loading control). *a.* Top panel, Northern blot of pS2 mRNA in pMV7pool and ERΔ3-3 clonal cells treated either with ICI 164-384, lanes 1 and 4 respectively; E2 ( $1 \times 10^{-8}$  M), lanes 2 and 5, respectively; E2 ( $1 \times 10^{-10}$  M), lanes 3 and 6, respectively. Bottom panel, Northern blot of GAPDH mRNA. *b.* Densitometric quantitation of pS2 expression, normalized for GAPDH, is expressed as fold stimulation by E2 over the ICI 164,384 baseline.

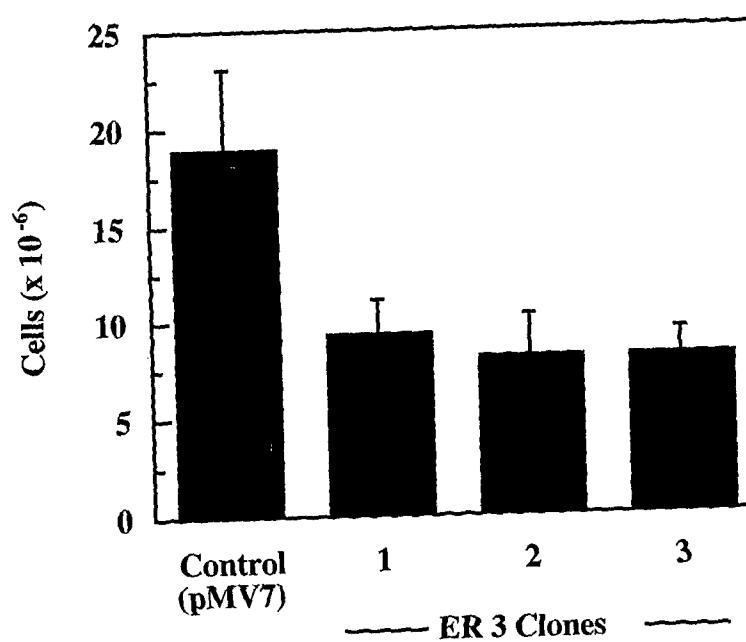
*ERΔ3 expression alters the growth properties of MCF-7 cells:*

During the initial selection in medium with FBS, the ERΔ3/pMV7 transfected clones grew much slower than the parental cells or the vector transfected clones. While it took 130 days for the ERΔ3/pMV7 cells to undergo 20 divisions (1 division every 6 days), it took only 83 days for the pMV7 clones (1 division every 4 days) (Fig. 15). It appeared from microscopic observations that the saturation density in the clones decreased; to further evaluate this difference, ERΔ3 clones and pMV7 controls were plated at 50% confluence in medium containing FBS with E2, maintained for 4 days beyond visual confluence, trypsinized and counted. Results in Figure 16 show that ERΔ3 clones reached a plateau of cell density that was only 50% of the cell number of the controls, perhaps indicating that cells expressing ERΔ3 are more sensitive to signals of contact inhibition. This was the first suggestion that the expression of ERΔ3 alters the tumorigenic phenotype of breast cancer cells toward behavior expected of normal cells.

**Figure 15**

**Figure 15*****Effect of ERΔ3 expression on growth of MCF-7 cells.***

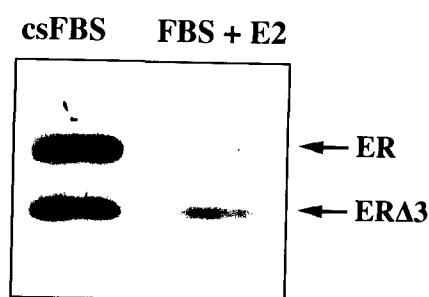
Clonal isolation of cells transfected with ERΔ3/pMV7 and pMV7 vector control in RPMI supplemented with FBS demonstrates the effect of ERΔ3 expression on growth of MCF-7 cells. Shown are the number of months in culture necessary for transfected cells to undergo 20 divisions, 83 days for the pMV7 controls (open square), and 130 days for the ERΔ3 expressing clones (closed diamond).

**Figure 16**

**Figure 16*****Effect of ERΔ3 expression on saturation density.***

$4 \times 10^6$  pMV7pool and ERΔ3-1,-2, -3 clonal cells were plated in 100mm tissue culture dishes in the presence of FBS and  $1 \times 10^{-8}$  M E2. Cells were maintained for four days beyond visual confluence, with a medium change every 2 days, detached and counted. Mean and standard deviation are calculated from three independent experiments. The difference in saturation density between each of the three ERΔ3-expressing clones and the pMV7pool was statistically significant (individual t-tests;  $p < 0.001$ ).

Shifting ER $\Delta$ 3 expressing cells into estrogen depleted serum (csFBS) stimulated their growth above that in FBS. This effect on growth was accompanied by a change in the ER $\Delta$ 3 to ER protein ratio. Western blot detection of estrogen receptors immunoprecipitated from an equal amount of protein of ER $\Delta$ 3 clone 2 cells, grown either in the presence of csFBS (depleted of estrogen) or E2 supplemented FBS, showed that there was more overall receptor protein in cells grown in medium with csFBS (Fig. 17). This gain was predominantly in the full length receptor, thus decreasing the ER $\Delta$ 3 to ER protein ratio. (Similar results were obtained with ER $\Delta$ 3 clone 1). These data indicate that when the culture conditions favor the predominance of ER $\Delta$ 3, as in FBS-E2 containing medium, cell growth is retarded. To maintain the ER $\Delta$ 3 to ER ratio in favor of the transgenic protein, all further experiments were carried out on cells grown in medium with FBS and estradiol, unless otherwise indicated. In contrast, for daily cell maintenance the clones were kept in medium supplemented with csFBS; otherwise due to their slow growth, the availability of cells for experimental analysis was limited.

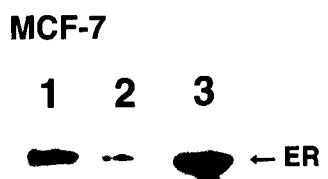
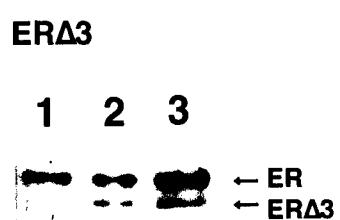
**Figure 17**

**Figure 17*****Effect of E2 on the relative ERΔ3 to ER protein level.***

Protein (400 ug) extracted from ERΔ3-2 cells plated at 4x10<sup>6</sup> cells per 100 mm dish and grown in either csFBS or FBS supplemented with 1x10<sup>-8</sup> M E2 for 72 hrs. was immunoprecipitated with rabbit anti-ER antibodies, and analyzed by western blotting using the H226 antibody as described.

***E2 regulation of ER protein:***

Given published observations that estrogen inhibits the expression of its own receptor in a negative feedback loop, and our own findings that ER expression is substantially reduced when cells are grown in E2 supplemented FBS, we determined the effect of ERΔ3 expression in the clones on estrogen regulation of ER. ERΔ3 clone 1 and parental MCF-7 cells ( $2 \times 10^6$  cells per 100 mm dish) were either treated with tamoxifen ( $1 \times 10^{-6}$  M) in 10% csFBS, E2 ( $1 \times 10^{-8}$  M) in 10% FBS, or left untreated and grown in 10% FBS for 24 hrs. As demonstrated by others, tamoxifen stimulated the expression of ER (Fig. 18, a, compare lanes 1 and 3), while E2 substantially reduced it in the control MCF-7 cells (Fig. 18, a, compare lanes 1 and 2). In contrast, whereas the stimulatory response to tamoxifen was retained in the ERΔ3 expressing cells (Fig. 18, b, compare lanes 1 and 3), estrogen inhibition of ER was almost completely abolished (Fig. 19, b, compare lanes 1 and 2). Although the mechanism by which ERΔ3 interferes with this estrogen dependent regulation remains to be determined, these findings demonstrate that it can not only abrogate the classic stimulatory effects of this hormone, such as pS2, but can also efficiently suppress its inhibitory actions.

**Figure 18***a.**b.*

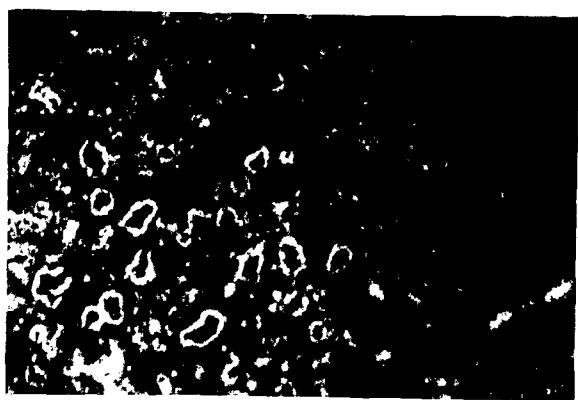
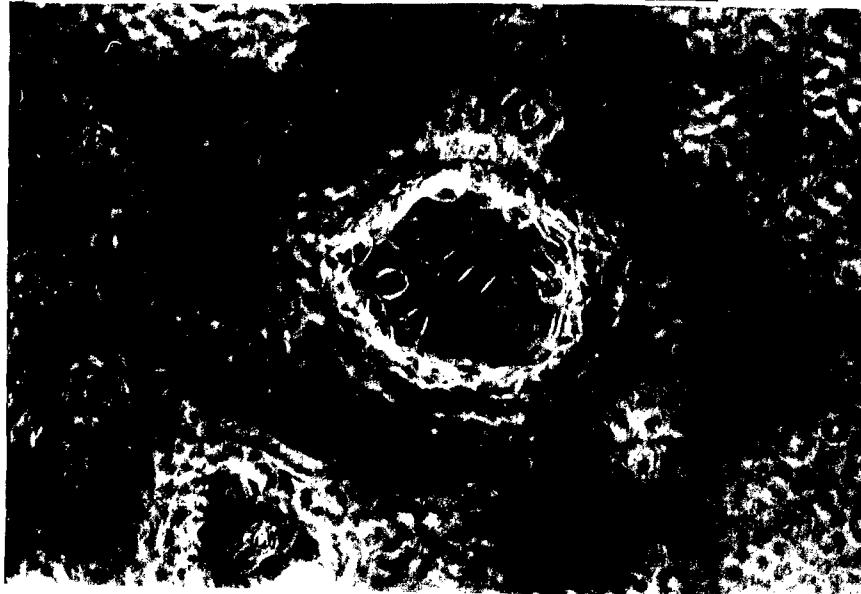
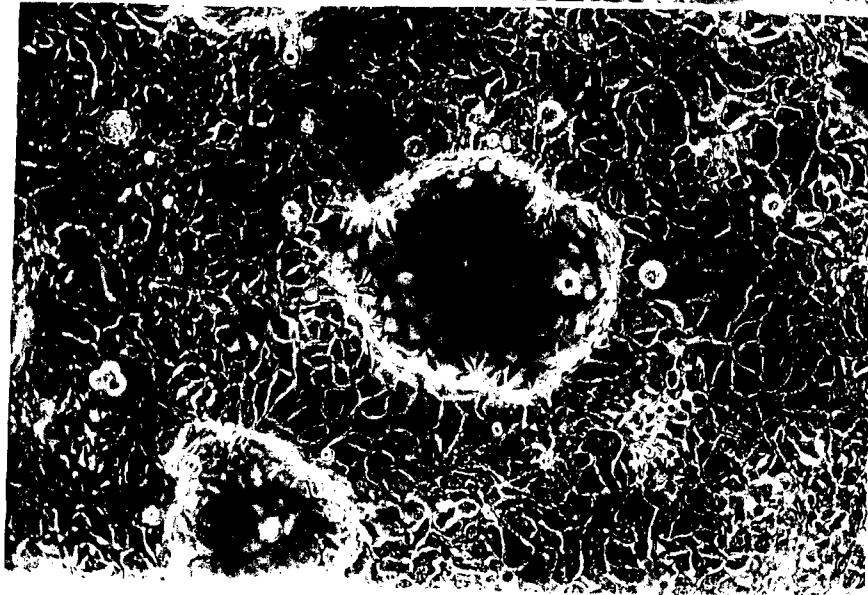
**Figure 18*****Effect of ERΔ3 expression on estrogen regulation of ER protein.***

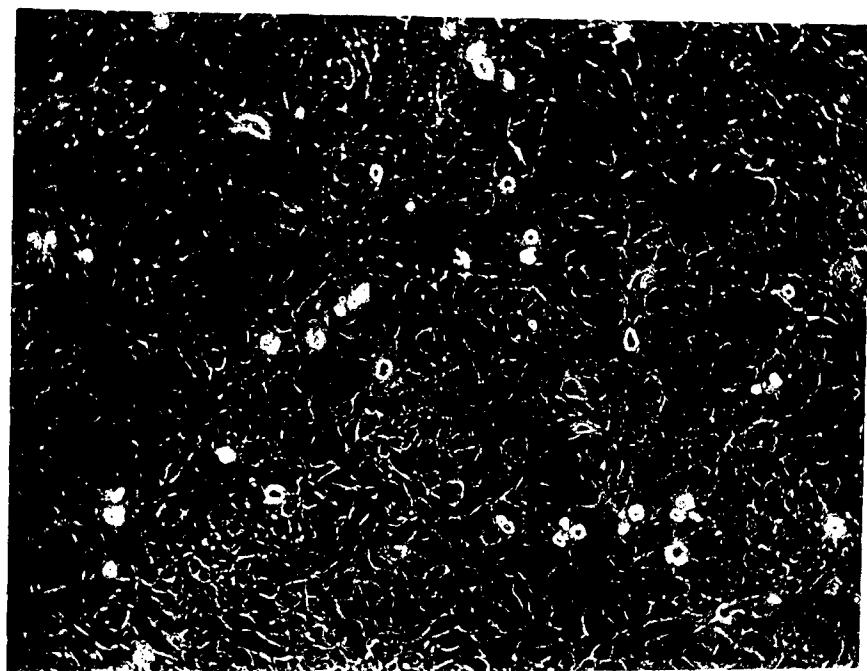
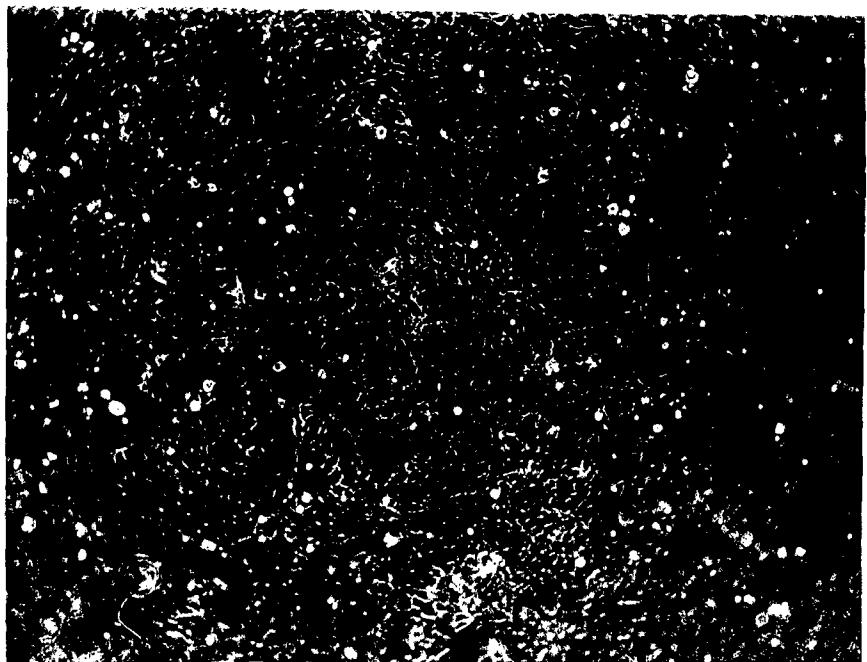
Protein (400ug) extracted from MCF-7 and ERΔ3-1 cells, plated at  $2 \times 10^6$  cells per dish and either treated with tamoxifen ( $1 \times 10^{-6}$  M) in 10% csFBS, E2 ( $1 \times 10^{-8}$  M) in 10% FBS or left untreated and grown in 10% FBS for 24 hrs, was immunoprecipitated with rabbit anti-ER antibodies, and analyzed by western blotting using the H226 antibody as described. *a.* MCF-7 cells: untreated (lane 1) or treated with E2 (lane 2) or tamoxifen (lane 3). *b.* ERΔ3-1 cells: untreated (lane 1) or treated with E2 (lane 2) or tamoxifen (lane 3).

*Changes in cell morphology of ERΔ3 expressing cells:*

Several studies have demonstrated that normal glandular epithelial cells in culture form multicellular secretory domes (120, 121). This *in vitro* marker of differentiation, characterized by polarized secretion of fluid and proteins inward (towards the bottom of the tissue culture dish), can be easily observed by light microscopy. Normal mammary epithelial cells have been shown to secrete milk proteins, such as  $\beta$ -casein, into the lumens of such domes in response to lactogenic hormones (19).

Several ERΔ3 expressing clones, when confluent, formed such polarized multicellular domes, with a lumen. In contrast, the parental MCF-7 and pMV7 control cells looked simply multilayered and overgrown when maintained at confluence. Although occasionally we observed a few swollen cells in these control cultures, possibly containing secretory material, such cells did not organize into the large secretory structures observed in the ERΔ3 expressing cells. An example of dome formation by ERΔ3-2 cells is shown in Fig. 19; a low magnification demonstrates the high density of these domes (Fig. 19a), and a higher magnification shows the top and bottom of one such multicellular dome (Fig. 19, b and c respectively). A comparably confluent culture of the pMV7 control cells (Fig. 19, d and e) shows no evidence of such differentiation.

**Figure 19***a.**b.**c.*

**Figure 19***d.**e.*

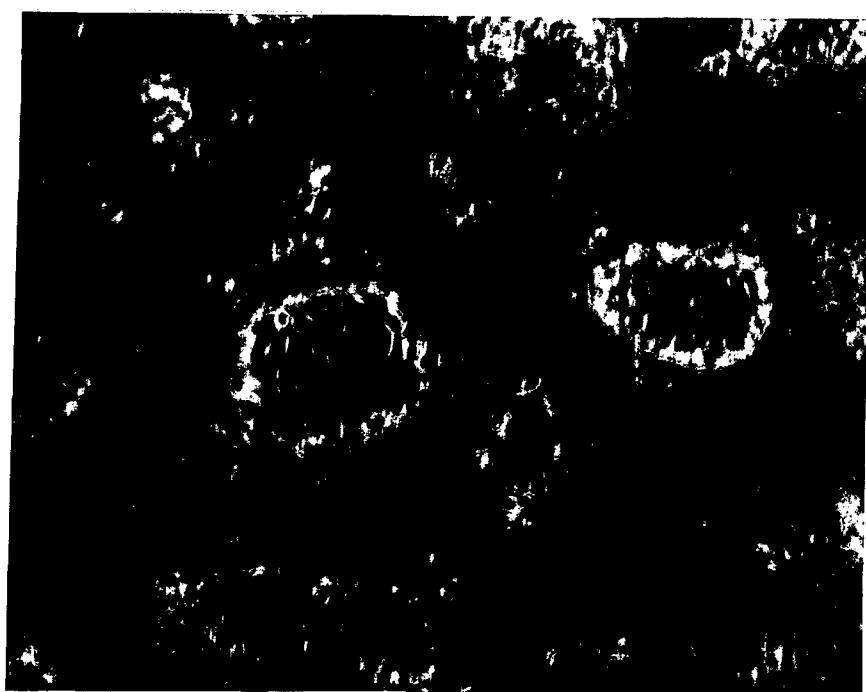
**Figure 19***Morphology of confluent ERA3 expressing cells and pMV7 controls: dome formation.*

$4 \times 10^6$  ERA3-2 and pMV7pool cells were plated into 100 mm dishes and cultured beyond confluence for 5 days. *a.* Density of dome formation by ERA3 expressing cells shown at a 40x magnification. *b.* High magnification (200x) view of the top of one such multicellular dome. *c.* High magnification (200x) view of the bottom of one such multicellular dome with an “empty” lumen. Surrounding cells growing in a monolayer on the dish marking the focal plane of the image. *d.* Confluent pMV7pool cells (200x magnification) showing no evidence of doming. *e.* Confluent pMV7pool cells (100x magnification) showing no evidence of doming.

When doming ERΔ3 cells were cultured for several days beyond confluence, a novel phenotype was observed. The cells in contact with the substratum (at the boarder of the elevated dome) developed cytoplasmic projections, extending underneath the fluid filled lumen. Such "feet" appeared to migrate from the circumference of the dome until they came in contact with each other, thus closing off the dome at the bottom (Fig. 20). Surprisingly, these large, multicellular "gland"-like structures, were able to dissociate from the plate and float in the medium as round balls of polarized, secretory cells surrounding a fluid filled lumen. In order to assess their three dimensional structure using confocal microscopy live spheres were incubated with BCECF-AM (2',7' -bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxyethyl ester), a pH indicator which diffuses into cells and is cytoplasmically modified to BCECF, a fluorescent, non-diffusible analog. Inspection of one such labeled sphere sectioned through the top, middle and bottom (Fig. 21, a-c respectively) demonstrates that a single layer of polarized epithelial cells containing multiple cytoplasmic secretory vesicles, surround a cell free lumen.

**Figure 20**

*a.*



*b.*

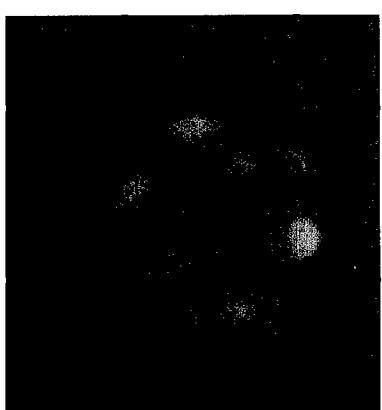


**Figure 20***Morphology of confluent ERΔ3 expressing cells: dome to sphere transition.*

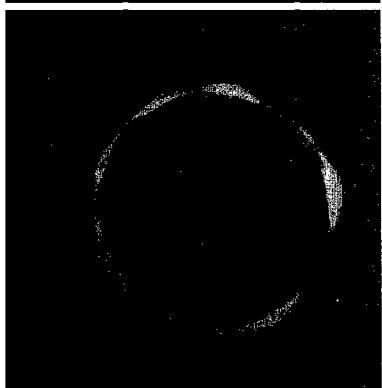
$4 \times 10^6$  ERΔ3-2 and pMV7pool cells were plated into 100mm dishes and cultured beyond confluence for 5 days. *a.* Top view of a field of domes formed by ERΔ3-2 cells (200x magnification). *b.* Bottom view of the same field shows cytoplasmic projections at the base of the domes.

**Figure 21**

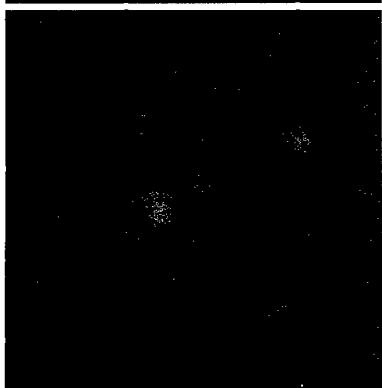
*a.*



*b.*



*c.*



**Figure 21*****Sphere formation by ERΔ3 expressing cells.***

ERΔ3-1 cells were cultured beyond dome formation to generate free-floating spheres. Spheres were collected, washed with PBS and stained with BCECF-AM for 30 min. at 37°C. Fluorescent confocal microscopy demonstrates the morphology of one sphere (approximately 150um in diameter) in panels a-c (~0.5 um thick sections). *a.* Top view shows the monolayer of cells in the sphere. *b.* Middle of sphere shows an empty lumen. *c.* Bottom cut shows the monolayer of cells in the sphere. Secretory vesicles are observed in some cells seen in *a* and *c*.

To determine if the ERΔ3 expressing cells in these free-floating spheres retain the ability to re-attach to a substratum and proliferate, or if they are in fact terminally differentiated, spheres were collected, allowed to settle, and replated onto a new tissue culture dish in fresh medium. Although a substantial number of spheres as well as cell debris remained in the medium, within two days of plating, some spheres attached and islands of growing cells were seen on the dish. When cultured, these cells formed large domes, and later spheres, without reaching confluence. No changes in the expression of ERΔ3 protein were observed, as determined by western blotting. These findings suggest that a subset of spheres contain cells with the capacity to proliferate in a monolayer culture. However the ability of such sphere derived cells to dome prior to confluence, suggests that a selection of this phenotype may have occurred.

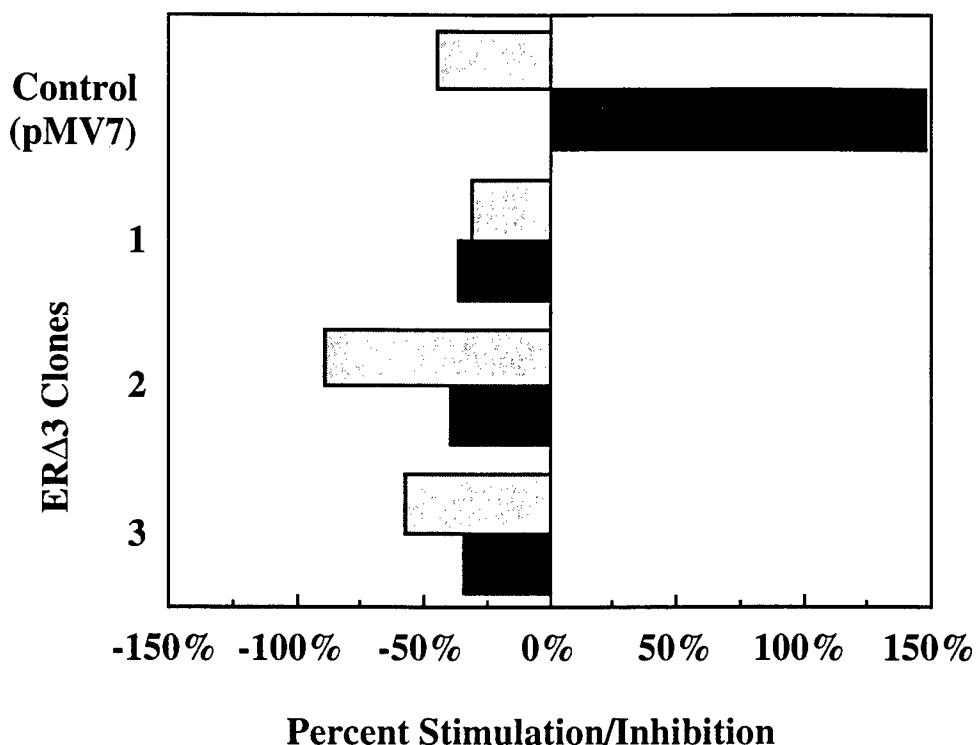
To date such organization of epithelial cells into secretory glandular structures *in vitro*, has only been demonstrated with normal mammary cells cultured in a biological basement matrix, such as matrigel (19). The surprising ability of several ERΔ3 clones, to organize into such secretory spheres, and retain their three dimensional architecture floating in medium, is the first demonstration of this phenomenon occurring in the absence of matrix. In addition, given that this "gland"-like phenotype is normally observed in non-transformed mammary epithelial cells, its occurrence in the MCF-7 cancer cells expressing ERΔ3, although only associative, suggests that the presence of this receptor isoform may

allow breast cancer cells to enter a pathway of differentiation usually tightly coupled to extracellular matrix signaling. Since cancer cells rarely maintain this fully differentiated morphology, the presence of both domes and spheres in ERΔ3 clones, not observed in either the parental MCF-7 or the vector control cells, suggests that some ERΔ3 expressing cells may have initiated and became responsive to signals of differentiation.

***ERΔ3 attenuates the tumorigenic phenotype of MCF-7 cells:***

The ability of cells to exhibit anchorage-independent growth correlates with their tumorigenicity *in vivo* (122). Consequently, we examined the effect of ERΔ3 expression on the anchorage-independent growth of MCF-7 cells (Fig. 22). As shown by others, E2 increased the ability of parental MCF-7 cells (and of the pMV7 control cells) to form colonies in soft agar. In contrast, estradiol treatment of ERΔ3 expressing clones grown in soft agar not only failed to stimulate colony formation, as observed in the controls, but inhibited it to below baseline levels. The magnitude of these reductions was comparable to that obtained by treatment with the anti-estrogen, tamoxifen, which, as expected, inhibited colony formation of both ERΔ3 expressing cells, and control pMV7 and parental MCF-7 cells (Fig. 22). Studies of ERΔ3 function as a dominant negative receptor predict that, at the relative ratios of ERΔ3 to ER expressed in the clones, estrogen responses should be attenuated by only 30%-50% (110), and not abolished completely or reversed as we have observed. As such, these data, taken together with similar findings of estrogen regulation of pS2 in these cells,

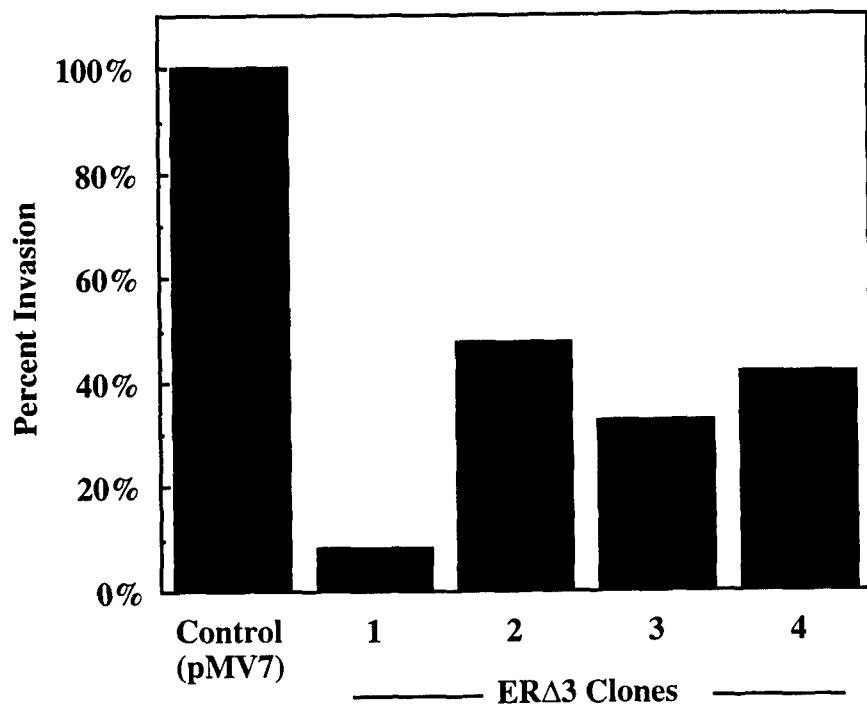
indicate that ERΔ3 is not only a dominant negative receptor but may have an additional role in estrogen signaling independent of ER; and that its presence, *in vivo*, may reverse the tumorigenic phenotype of breast cancer cells.

**Figure 22**

**Figure 22*****Anchorage-independent growth of ERΔ3 and pMV7pool cells.***

Low melt agarose (Seaplaque, 1% in lower and 0.4% in upper layer) was prepared in DMEM with insulin (5ug/ml) and 10% FBS (+/-  $1 \times 10^{-8}$  M E2 or +/-  $1 \times 10^{-6}$  M tamoxifen). To assess anchorage independent growth, pMV7pool and ERΔ3 clones 1, 2, and 3 cells mixed with agarose (upper layer) were distributed on top of 5ml of lower DMEM/agarose layer, grown for 2 weeks and scored for colony formation. Colonies were scored in 1/4 to 1/2 of each dish. The results are the mean of duplicate determinations. Stimulation or inhibition by E2 and tamoxifen is expressed as percent of colonies in agarose containing medium with FBS. The cloning efficiency of the pMV7pool cells under control conditions (medium with FBS alone) was 6.5%.

Finally, the metastatic properties of tumor cells are integrally linked with their ability to invade surrounding extracellular matrix. Several proteolytic enzymes shown to be involved in cancer cell invasion (such as plasminogen activators, collagenase IV, and cathepsin D), are regulated by estrogen (16, 48-52, 123-126). To assess the effect of ERΔ3 expression on breast cancer cell invasion of host tissue, pMV7 control and ERΔ3 clone-1, 2, 3, or 4 cells were grown in the presence of estrogen, metabolically labeled with <sup>125</sup>IUDR for 24 hours and each inoculated onto eight wounded and resealed chick embryo chorioallantoic membranes (CAMs), an *in vivo* vascularized tissue. Following a 24 hour incubation, the CAMs were washed with PBS (and washes collected), excised and incubated with trypsin-EDTA, to remove any surface attached non-invasive cells, without disrupting the cells that have infiltrated the connective tissue. By measuring the amount of radioactivity remaining in the CAMs after trypsinization (which reflects the number of cells that have invaded the CAM) and comparing it to the total amount of radioactivity (total number of cells inoculated is determined by the sum of radioactive counts in the PBS wash and CAMs before trypsinization), the percent invasiveness was calculated. We determined that, compared with the parental MCF-7 cells or pMV7 vector control cells, the ability of ERΔ3 expressing clones to invade the CAM was reduced by 52-79% (Fig. 23). This substantial reduction in invasiveness of ERΔ3 expressing cells suggests that the presence of this receptor isoform *in vivo*, may suppress malignant potential of cancer cells.

**Figure 23**

**Figure 23*****Effect of ERΔ3 expression on in vivo invasion.***

Eight replicate chick embryo chorioallantoic membranes (CAMs) were inoculated with  $3 \times 10^5$  cells per CAM of pMV7pool or ERΔ3 clones 1, 2, 3, or 4 cells grown in the presence of  $1 \times 10^{-8}$  E2 for 72 hrs. and labeled with 0.2 uCi/ml of  $^{125}\text{I}^{\text{UdR}}$  for the last 24 hrs. (specific activity 0.1 to 0.2 cpm/cell). Preparation of CAMs for inoculation and quantitation of invasion was as described and CAMs were resealed prior to inoculation for 22 hrs. Compared to the invasion by the control pMV7pool cells, invasion by ERΔ3-1 clone was significantly reduced,  $p < 0.01$ ; the reduction of invasion of the 3 additional clones, ERΔ3-2, -3, and -4 approached statistical significance ( $0.1 > p > 0.05$ , Mann-Whitney U test statistic).

## DISCUSSION

Estrogen exerts a tightly controlled effect on growth and differentiation of normal breast tissue during puberty (11-16). In an adult female, the mammary epithelium retains its ability to respond to changing circulating estrogen levels with controlled cyclical proliferation (11). However, the same hormone acts as a potent mitogen in breast cancer, potentiating its uncontrolled growth and invasion (1, 2). This dichotomy suggests that, during oncogenic transformation, mammary epithelial cells may undergo changes leading to aberrant or inappropriate estrogenic responses. The evidence presented in this thesis is the first demonstration that a selective loss of a non-DNA binding estrogen receptor isoform (ERΔ3) may contribute to the altered behavior of cancer cells in response to E2. This conclusion stems from two independent observations, one showing that the median ratio of ERΔ3 to ER in normal mammary cells is more than two logs greater than in breast tumors and tumor cell lines, and the second providing evidence that re-expression of ERΔ3 in an estrogen responsive breast cancer cell line, at relative levels comparable to that present in normal mammary epithelium, results in a shift of the cells towards a normal phenotype and a substantial reduction of their malignant potential.

The suggestion that a change in estrogen receptor RNA splice choice occurs in breast cancer is not unusual given the substantial literature documenting multiple exon skipped ER mRNAs in breast tumors (91-114). However, our findings demonstrate that a specific

loss of ERΔ3, and not gain, as has been proposed for several other ER isoforms, occurs in breast cancer. Research into the underlying causes of cancer has identified multiple genetic abnormalities in genes encoding proteins which control normal cell growth, differentiation and death (2). ER is no exception; loss of appropriate ER function has been documented with several mutations and gene inactivation (2, 93); but by enlarge one of the most common changes in breast cancer is its level of expression, much higher than seen in normal mammary epithelium (2).

Our finding that the ratio of ERΔ3 to ER is substantially lower in breast cancer than normal mammary tissue (Fig. 7), suggests that regulation of splicing of the precursor ER RNA is altered during oncogenic transformation. Alternative splicing of multiple RNA's has been demonstrated to occur in both a tissue and cell type specific manner (83, 127-129), and is believed to be one of several mechanisms that have evolved in eukaryotic cells to generate diversity. RNA splicing is a complex process involving the assembly of a catalytic spliceosome consisting of both protein and RNA subunits (129-132); intronic sequences are excised via the formation of an intermediate lariat, and coding exons are rejoined (129-132). How a cell directs the splicing machinery to the correct 5' and 3' splice sites, and what distinguishes different donor and acceptor sites of multiple exons in a single RNA is currently under investigation. Recent findings suggest that regulated splice choice involves not only the presence of appropriate splice factors, whose expression may be tissue or cell type specific (129, 132), but also on their relative

abundance in a cell and affinity for a particular splice site in its native RNA context (129, 131). In addition, as shown with the dramatic example of sex determination in *Drosophila Melanogaster*, both repression and activation of splice site by members of the SR family (serine/arginine rich phosphoproteins) belonging general splicing factors can regulate splice choice (129, 131, 133). By binding to a putative exon acceptor sequence such regulatory proteins either antagonize or recruit the assembly of the spliceosome leading to either exclusion or inclusion of a particular coding exon (129, 131, 133). Given the molecular complexity of such regulation it is easy to envision how a reduction of the ER $\Delta$ 3 splice isoform can occur in cancer. If the skipping of exon 3 in normal cells, and consequently the preferential use of the exon 4 splice acceptor site, is dependent either on the presence of an activator or repressor, or on the ratio of particular splice factors, a carcinogenic event leading to a change in expression of any such proteins can lead to a loss of ER $\Delta$ 3 expression. Given this change in the regulatory pathway of ER RNA maturation, the relative contribution of ER $\Delta$ 3 mRNA, and consequently protein, would be reduced, thus establishing permissive conditions for uncontrolled estrogen signaling in cancer.

The identification of ER-mRNA splice isoforms in breast tumors, initiated a series of studies as to their putative role in cancer (91-114). Possibly the isoform that appeared most relevant was ER $\Delta$ 5, missing ligand binding domain and predicted by *in vitro* studies to function as a constitutive transactivator (97, 98). Since progesterone receptor is regulated by the E2/ER pathway, it was predicted that the

ER-/PR+ breast cancer subpopulation will be enriched in the ERΔ5 isoform. It was also anticipated that this isoform may be responsible for the emergence of cancers resistant to tamoxifen treatment. However additional extensive analyses of its *in vivo* expression and attempts to demonstrate its function in transfection experiments (99) failed to lend support to the original hypotheses. The functional studies of another splice isoform, the ERΔ7, have also provided conflicting results. Present in normal and tumor breast tissue, and missing the carboxy terminal end of the hormone binding domain, ERΔ7 was shown to function as a dominant negative receptor in one study (95), and to have no effect on estrogen signaling in another (110). It is likely that as with other proteins, whose diversity is also generated by alternative splicing (128), a functional role for these splice isoforms of ER will be identified in estrogen target tissues other than the breast.

Overall our results provided convincing evidence for the relative loss of ERΔ3 in breast cancer. In order to determine whether this change was associated with early stages of breast cancer or was the result of disease progression, a comparative analysis was done on patients grouped according to their disease stage. Several indexes, such as the size of the tumor and the presence or absence of metastatic disease in the lymph nodes, as well as the menopausal status (since breast cancers in young women are considered more aggressive) were included in the assessment. We also compared patients whose tumors were identified as ER-negative by a routine ligand binding assay, commonly considered a more aggressive

phenotype (1, 29), to their ER-positive counterparts. The analysis showed no difference in ERΔ3/ER ratio between small tumors, or tumors that have not spread to lymph nodes, and more advanced tumors (larger, with lymph node involvement), suggesting that the loss of ERΔ3 may be an early event in carcinogenesis. Furthermore, we did not identify a difference between the ERΔ3 to ER ratios of pre- versus post- menopausal women. However, significantly lower ratios (0.05,  $p<0.001$ ) were found in tumors with  $ER < 5$  fmole/mg, clinically deemed ER negative. It is possible that a further drop of ERΔ3 may be associated with disease progression and with a cancer phenotype considered more aggressive and less differentiated. This severe under-representation of ERΔ3 was not due to the low overall ER level, since normal breast epithelial cells that express a comparably low amount of receptor have a substantially greater (3.4) ERΔ3 to ER ratio (Fig 7). The comparison indicates that the high relative expression of ERΔ3 observed in normal cells (Fig. 6 and 7), is not an exclusive property of cells containing a small number of receptors, underscoring the specificity of the expression of this splice isoform in normal mammary glands.

The presence of estrogen receptor in normal breast cells warrants further comment. Most studies of ER in normal human mammary tissue have used relatively insensitive immunohistochemical or biochemical techniques (3, 11), which were capable of detecting ER protein only in a subset of luminal epithelial cells, hence classifying all other cells in normal breast tissue, as estrogen receptor negative (3). In contrast, our study, using RT-PCR,

demonstrated the presence of ER-mRNA both in luminal and basal/myoepithelial cells of the normal breast epithelium (Fig. 6 and 7). It is interesting to note that basal epithelial cells, which express the highest relative level of ERΔ3, exhibit a low mitotic activity (20, 25). In fact, estrogen stimulated growth of mammary epithelium is most often observed in luminal cells of the ducts and lobules (20, 25).

Our analysis also demonstrated ER mRNA and protein in normal mammary fibroblasts (Fig. 7 and 8). The presence of both ER mRNA and protein in these cells is consistent with previous reports which demonstrated that mammary fibroblasts are estrogen responsive (13, 27). Furthermore, recent studies using similar sensitive RT/PCR or amplified immunocytochemistry techniques, identified ER in other cells considered historically devoid of ER. Among these are vascular endothelial cells, osteoblasts, as well as several neuronal cell types (107, 134-136). These data indicate that as shown in the epithelium, estrogen may exert its effect on the mammary stroma directly via the ER present in these cells.

A critical obstacle in the study of ER splice isoforms has been the identification of proteins endogenously translated from the spliced mRNAs. Only one other isoform (ERΔ5) has been demonstrated to date as an *in vivo* translated protein (98). This protein, with a molecular weight of 42kD, was detected by western blotting, in cells (BT20) previously found to contain a substantial amount of ERΔ5-mRNA (98). In the case of ERΔ3, the difficulty lying

in the fact that cells with greater overall ER concentration (such as breast cancer cells) have an extremely low ratio of the isoform, while cells with high ER $\Delta$ 3/ER ratio, such as normal epithelial cells, have extremely low overall levels of ER. This difficulty notwithstanding, data presented in this thesis demonstrates that in addition to its stable expression as a transgene in breast cancer cells, the native ER $\Delta$ 3 protein is expressed in MCF-7 cells, at a level expected from the relative content of its mRNA (Fig. 9,12,13). Given that our attempt to generate ER $\Delta$ 3 specific antibodies was unsuccessful, endogenous ER $\Delta$ 3 protein was identified by immunoprecipitation with one anti-ER specific antibody and western blotting with another (Fig. 13). Due to the small difference in molecular weight between ER $\Delta$ 3 (61 kDa) and ER (65 kDa), a long running time (10 hrs.) at a high voltage (200 Volts) was necessary in order to sufficiently separate the two receptors on an SDS-PAGE gel. Such separation yielded a major band at the expected (65 kDa) molecular weight and a very minor band running faster and matching a molecular weight of 61 kDa (Fig 13, lane 2). In addition, since ER can exist in both a hypophosphorylated and hyperphosphorylated state (28, 67, 137), to exclude the lower molecular weight (ER $\Delta$ 3) band as the hypophosphorylated ER, immunoprecipitated estrogen receptors from MCF-7 cells were treated with a phosphatase, and a clone with easily detectable ER $\Delta$ 3 protein was used as a molecular weight control (Fig. 13, lanes 3 and 4). This experiment not only confirmed the identity of the ER $\Delta$ 3 protein, due to the co-migration (both before and after dephosphorylation) of the endogenous ER $\Delta$ 3 protein in the MCF-7 cells with the stably expressed ER $\Delta$ 3 in the clonal cells, but suggested

that like ER, ERΔ3 is a phosphoprotein *in vivo*, and may be similarly activated by a non-classic, phosphorylation dependent mechanism. The shift in electrophoretic mobility of ERΔ3 which followed dephosphorylation was of somewhat greater magnitude than could be predicted. However, since no smears or small fragments were observed either in western blot detection or in Ponceau S stain in lanes treated with CIP, we conclude that this shift was not caused by proteolytic degradation of ERΔ3 during the dephosphorylation. Although several factors, such as a change in conformation or more extensive state of phosphorylation of the ERΔ3, may account for the enhanced mobility of the dephosphorylated form, our current experiments cannot provide conclusive explanation for this observation.

To be able to visualize the ERΔ3 proteins, each lane in the western blot had to represent receptors immunoprecipitated from 1 mg of total protein. In the instance of MCF-7 cells, the high level of ER (~200 fmol/mg of protein or ~12,000 receptors per cell) (30), as compared to normal breast tissue, was sufficient to detect ERΔ3 protein (which accounts for approximately 5% of total estrogen receptor protein in these cells). The very low abundance of ER in normal mammary cells as well as their limited availability, precluded direct analysis of ER and ERΔ3 proteins in these cells. However, the finding that in MCF-7 cells the ERΔ3 to ER protein ratio reflects that of the corresponding native RNAs (Fig. 9, 12, 13), makes the likelihood of a similar correspondence in normal cells highly plausible.

From published evidence, of ERΔ3 inhibition of estrogen activation of an ERE-CAT reporter under conditions of transient transfection (110), it was expected that ERΔ3 may interfere with estrogen stimulation of endogenous gene expression through a dominant negative mechanism. Consequently, estrogen responsiveness was assessed in MCF-7 cells expressing ERΔ3 at a relative ratio of ERΔ3 to ER not exceeding that observed in the normal mammary epithelium (Fig. 6 and 12). A gene, considered to be regulated by the classical E2-ER interaction with an ERE in its promoter, is the pS2 gene (80).

As expected, ERΔ3 suppressed estrogen induction of pS2 mRNA in all ERΔ3 expressing clones. The published *in vitro* analysis predicted that at the ratio of ERΔ3 to ER present in our clones, a 30% reduction of estrogen stimulated pS2 expression should be observed (110). Surprisingly, we observed an almost complete abolition (more than 90%) of E2 induction of pS2-mRNA, as compared to that in the control MCF-7 cells (Fig. 14). This magnitude of inhibition was much greater than predicted by the action of ERΔ3 as a dominant negative receptor, suggesting several possible explanations. The function of ERΔ3 as a dominant negative receptor assumes that given a random dimerization interaction, the formation of ERΔ3/ER heterodimers will decrease the amount of ER available for ER/ER homodimerization, thus attenuating estrogen induction of an ERE responsive gene (at a 1:1 ratio of ERΔ3 to ER a 50% maximal reduction of E2 stimulation of gene expression is expected).

However, the action of ER is not only regulated by its binding to DNA, but also its interaction with accessory proteins, whose expression in a cell may be limited (76-79). In this instance ERΔ3/ER or ERΔ3/ERΔ3 dimers may bind to such co-activators, thus reducing their availability to interact with ER, resulting in a further drop in the magnitude of estrogen stimulated transactivation. Alternatively, ERΔ3 may actively inhibit gene expression by interacting with other transcription factors, via a non-DNA binding domain dependent mechanism (46). Future studies will provide the distinction between these pathways and delineate the mechanism of ERΔ3 action.

We have also demonstrated that estrogen mediated down regulation of its own receptor is efficiently abrogated by the presence of ERΔ3. As expected, in response to E2 treatment a dramatic reduction in ER protein was observed in the parental MCF-7 cells, but not in the ERΔ3 expressing clones (Fig. 18). In contrast, the increased level of ER in tamoxifen treated cells was similar in both vector control and ERΔ3 clones (Fig 18), suggesting that the expression of this receptor isoform can specifically inhibit estrogen action, without affecting the response to tamoxifen. The mechanisms by which estrogen regulates ER expression are complex, involving a decrease in ER gene transcription (through a region of DNA which does not contain a classic ERE), as well as destabilization of its mRNA and an increased protein turnover (30, 118, 138-141). In contrast, tamoxifen has been found to increase the level of ER protein in MCF-7 cells, without affecting the steady state levels of its mRNA (30). The role of ER, and consequently ERΔ3 in these molecular pathways

is still unclear. However, the ability of ERΔ3 to interfere not only with the stimulatory estrogen regulation of gene expression, but also its inhibitory actions, underscore the importance of this receptor isoform in controlling responses to hormone *in vivo*.

The suggestion that ERΔ3 may limit the magnitude of estrogen action *in vivo* when hormone levels are high, is underscored by the demonstration of an autoregulatory loop in the clones containing ERΔ3. In these cells exposure to estrogen, after continuous growth in csFBS, can shift the complement of estrogen receptors from mostly ER to predominantly ERΔ3 (Fig. 17). This is achieved by a more pronounced down-modulation of ER than of ERΔ3 proteins and may occur via several mechanisms, including increased mRNA and protein turnover (30, 118, 138-141)(43, 44). If a similar mechanism of auto-regulation exists in endogenous mammary tissue, then during periods of peak estrogen availability, a rise in the ERΔ3 to ER protein ratio may protect breast tissue from over-stimulation. Thus, oncogenic transformation of breast cancer cells, resulting in a permanent selective reduction in ERΔ3 expression, would lead to a disruption of this response, promote unchecked estrogen action, and establish permissive conditions for further carcinogenic events.

Our assessment of several features that distinguish transformed from normal cells in ERΔ3 expressing clones, revealed that these cells not only exhibit a considerable reduction in their tumorigenic phenotype (Fig. 22 and 23), but also acquire growth and morphologic characteristics consistent with normal mammary cells

(Fig. 15,16,19, 20, 21). As noted, in the ERΔ3-transfected clones the relative level of this isoform is highest in the presence of estrogen. It is interesting that such estrogen treatment also causes a marked reduction of growth and, more importantly, a much lower saturation density (Fig. 15 and 16). These effects are specific to the ERΔ3 isoform, since a similar transfection of full length ER into either MCF-7 or T47D cells (which are also ER-positive), did not reduce their proliferative response to hormone (142). Although not yet examined, a testable hypothesis is that a dominant negative receptor interferes with estrogen stimulation of genes critical for growth regulation, such as cyclin D1, myc, and the fos/jun family of transcription factors (33, 37). In addition, estrogen stimulation of several growth factors and their receptors (1, 2, 4, 28, 30, 32, 33, 35), may be decreased in cells containing ERΔ3, possibly contributing to the lower saturation plateau.

The ability of ERΔ3 expressing clones to form secretory domes (Fig. 19 and 20) further indicates that these cells have acquired properties of normal mammary epithelium in culture. This differentiated phenotype of epithelial cell polarization, is marked by the formation of junctional complexes, such as adherens and tight junctions (19). Although MCF-7 cells have been reported to express some of the components of such junctions, and the expression of at least one, E-cadherin, is modulated by estrogen (143), it is unclear whether these proteins organize into functional complexes in these cells. Perhaps, the expression of ERΔ3 in these cells potentiates this differentiation by aiding the assembly of an adhesion belt (whose

contraction has been implicated in lumen formation during gland development (17), and tight junctions (necessary for polarized secretion) (17), leading to the observed doming morphology. A few studies have demonstrated that MCF-7 cells can form domes in response to tamoxifen treatment (144), however, during the course of our investigation, we did not observe this phenomenon. The existence of many variants of this cell line may be responsible for such different findings. Future analyses of signaling pathways of this receptor isoform may determine if tamoxifen and ERΔ3 share downstream molecular partners critical for such differentiation.

With continued culture the dome producing ERΔ3 cells yielded a novel phenotype characterized by secretory sphere formation (Fig. 21). To our knowledge, such alveolar organization has never been demonstrated in the absence of an exogenous extracellular matrix, either in normal or cancer cells. Studies of normal mammary epithelial cells grown in Matrigel, have demonstrated that the highly polarized epithelium of the differentiated spheres contains a basely located nucleus and an apical membrane which faces the lumen (19), mimicking the *in vivo* organization of a mammary lobule (11). Although not yet tested, it is likely that a similar orientation exists in the free-floating ERΔ3 expressing clonal spheres. Given that a loss of polarized epithelium and consequently, glandular architecture is a primary hallmark of cancer (11), it is striking that upon expression of ERΔ3 in breast cancer cells, we observe a reversal of this phenotype (Fig. 21), evidenced by spontaneous formation of secretory spheres. Moreover, these data suggest that expression of

ERΔ3 in MCF-7 cells may have initiated a pathway of differentiation which is integrally linked to matrix production and signaling. Further analyses of such cells may identify the link of ERΔ3 function with the downstream events that regulate mammary responses to extracellular matrix.

Concurrently with the observations that ERΔ3 expressing cells acquire a more "normal" phenotype, a significant reduction in the transformed phenotype of these cells was observed. We have shown that the presence of ERΔ3 interferes with the ability of breast cancer cells to invade the stroma of connective tissue *in vivo* (Fig. 22). The magnitude of the inhibitory effect (52-79%) suggests that ERΔ3 functions via a dominant negative mechanism. It is known that estrogen is necessary for MCF-7 tumor growth and metastasis in nude mice (122). Estrogen also stimulates the expression of several proteolytic enzymes (such as plasminogen activators, collagenase IV, cathepsin D), shown to be involved in cancer cell invasion (123-126, 145). It is likely that the presence of ERΔ3 will effectively interfere with E2 stimulation of these proteases, resulting in a reduced invasiveness of these cells.

Our assessment of estrogen stimulation of anchorage independent growth, an *in vitro* property predicting tumorigenicity, revealed that E2 not only fails to stimulate anchorage independent growth in ERΔ3 expressing cells, but similarly to the effect obtained with the anti-estrogen, tamoxifen inhibits it to below baseline levels (Fig. 23). Furthermore, although growth in soft agar in response to

E2 has been dramatically altered in ERΔ3 expressing cells, the reduced colony formation in response to tamoxifen was comparable in both clone and control cells (Fig. 23). These findings indicate that ERΔ3 specifically interferes with estrogen action, without affecting the interaction between an antiestrogen and ER. The magnitude of E2 inhibition of growth in soft agar cannot be explained purely on the basis of a dominant negative effect, since in most of the clones tested, the ratio of ERΔ3 to ER is not greater than 1 to 1. The suggestion that ERΔ3 acts through an additional pathway is further supported by published observation showing that transient co-transfection of ERΔ3 and ER proteins, at ratios comparable to those present in our clones, produced only a 30% inhibition of estrogen dependent transactivation (110), and is further supported by our findings of almost complete abolishment of E2 stimulation of pS2-mRNA in cells expressing ERΔ3, as discussed previously (Fig. 14). Since the total ER level in the clones is either equal to, or less than, that in the parental MCF-7 cells, the observed effect could not be due to the general over-expression of ER protein, shown by some to lead to E2 inhibition of growth (146, 147). Although, we have not yet investigated the mechanism of the suppressive signal transduction pathway of ERΔ3, as mentioned previously, it is likely that this receptor isoform, in addition to its dominant negative action, participates in the non-classic regulation of gene expression via protein/protein interaction with other transcription factors, recently shown to be both, independent of ER binding to DNA (148), and importantly, independent of the ER-DNA-binding domain (46).

Thus, by re-introducing ERΔ3 into estrogen responsive breast cancer cells, their transformed phenotype was either attenuated or, in some instances, reversed. In addition to our observations of a significant reduction of both anchorage dependent and independent growth, as well as a marked decrease in the ability of ERΔ3 expressing cancer cells to invade connective tissue *in vivo*, suggesting reduced tumorigenicity, the formation of domes and spheres by such clonal cells suggests that ERΔ3 may be a critical regulator of normal mammary gland differentiation.

Thus we have demonstrated a novel function for a non-DNA binding estrogen receptor isoform in breast biology. Relative high expression of this isoform in normal mammary tissue may provide a mechanism for attenuating estrogenic effects, and its reduction in breast cancer may lead to excessive, unregulated mitogenic action of this hormone. Our results indicate that, as with tumor suppressor WT1 (149, 150) and cell adhesion molecule CD44 (151), carcinogenic events in breast can lead to alteration of splice choice pathways, but unlike suggested for other ER isoforms (91-114), rather than being elevated in cancer, the relative ratio of this isoform is diminished. Although less likely, it is possible that ERΔ3 to ER ratio is variable in normal epithelial cells and that the cells with low ratios are more susceptible to carcinogenesis. Further studies of the mechanisms through which ERΔ3 exerts its effect will clarify its role in controlling E2 responsiveness in mammary cells. Identifying ways to re-direct the pathway towards enhanced expression of ERΔ3, or finding

alternative means of increasing its relative ratio, may provide a novel avenue for future breast cancer therapy.

## FUTURE STUDIES

The findings described in this thesis are the first demonstration that an estrogen receptor splice isoform may have an important function in estrogen regulation of normal and cancer breast tissue responses to hormone. Several avenues exist for future studies of ERΔ3 in mammary biology.

The analysis of endogenous tissue presented here, suggests that loss of ERΔ3 in breast cancer may be an early event during carcinogenesis. To further determine when this change in expression occurs, an *in situ* hybridization analysis can be undertaken to compare its expression in normal, hyperplastic (typical and atypical), DCIS, and cancerous breast tissue. This analysis will establish when during cancer development a loss of this receptor isoform occurs, and determine if ERΔ3 can be clinically useful as an early marker of oncogenic transformation.

In order to fully understand the mechanism of ERΔ3 action, a characterization of the function of ERΔ3/ER dimers needs to be undertaken. ERΔ3/ER heterodimer formation, in addition to exerting a dominant negative effect by interfering with ER/ER homodimer formation, may be able to bind to a half palindromic ERE sequences in the DNA, and thus regulate estrogen responsive gene expression. In addition, the effect of ERΔ3 on non-classic gene expression needs to be evaluated. Given findings that ER can activate AP-1 dependent gene expression, independent of binding to DNA and

the DNA binding domain (46), the role of ERΔ3 in such transcriptional regulation remains unclear and awaits further investigation.

In order to determine whether the function of ERΔ3 is influenced by similar molecular mechanisms as ER, its ability of bind the regulating hsps in the absence of ligand, and interact with co-activators (such as RIP140, ERAP140, TIF-2) needs to be evaluated. For instance, if a limited amount of co-activator is available in a cell, and its interaction with ER is necessary for efficient estrogen stimulation of gene expression, ERΔ3, by competing for such factors, would interfere with ER induction of gene expression.

Our data suggests that, like ER, ERΔ3 is a phosphorylated protein, *in vivo*. ER phosphorylation has been demonstrated to be an important mechanism of receptor activation in response to growth factor signaling, resulting in ER regulated gene expression. The residues critical for such activation are located in the amino-terminal A/B domain of the receptor and are conserved in ERΔ3. Consequently the importance of such phosphorylation in ERΔ3 function needs to be evaluated.

Our observations of a reduction in anchorage dependent and independent growth, and invasion, warrant further analyses. As mentioned in the text, several candidate genes that may contribute to such phenotypes are regulated by estrogen (1, 2, 4, 28, 30, 32, 33, 35, 123-126, 145). Consequently, to determine the molecular

mechanisms by which ERΔ3 exerts its effect of diminished growth and malignant potential, the expression of such estrogen regulated genes must be assessed.

We have observed that ERΔ3 expressing cells display a novel phenotype of sphere formation in culture. In order to conclusively demonstrate that such differentiation is indeed a consequence of the expression of this receptor isoform, ERΔ3 can be stably introduced into MCF-7 cells under the control of an inducible promoter. Using this system, an analysis of this differentiated phenotype can be carried out with induced versus uninduced cells, to establish a more causal relationship between ERΔ3 expression and sphere formation. To name a few further studies, such spheres can then be analyzed for polarity, using markers specific for the apical membrane and junctions, response to lactogenic hormones, as determined by secretion of milk proteins into the lumen, and the role of matrix molecules in sphere assembly, by use of blocking antibodies to matrix proteins and their receptors.

Finally, an exploration of the factors that govern splice choice during ER pre-mRNA maturation is critical. The carcinogenic event that results in a loss of ERΔ3 expression occurs along this ER-RNA splicing pathway. Consequently, by understanding the regulation of the alternative splicing which leads to ERΔ3 expression, we may identify the underlying cause of its under-representation in cancer cells. Since the pre-mRNA template is retained in the cancer cell, perhaps such studies will help identify a means of altering splice

choice in favor of ERΔ3 in cancer cells, thus establishing a new avenue for breast cancer treatment.

These are just some of the many necessary studies that need to be undertaken to delineate the complete role of ERΔ3 in breast biology, breast cancer and estrogen responsiveness.

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